



SZENT ISTVÁN UNIVERSITY

Animal Husbandry Science PhD School

**NEURONAL DISEASE MODELLING WITH RABBIT
AND HUMAN STEM CELLS**

Thesis for Doctoral degree (PhD)

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Gödöllő

2017

The PhD program

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Discipline: Animal Husbandry Science

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ABBREVIATIONS

3-NT	3-nitrotyrosine
AA	ascorbic acid
A β	β -amyloid
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
ADAM	a disintegrin and metalloproteinase
AEBSF	4-(2-Aminomethyl)benzenesulfonyl fluoride hydrochloride
AICD	APP intracellular domain
AKT	serine/threonine protein kinase
ALS	amyotrophic lateral sclerosis
AP	alkaline phosphatase
APH1A	Aph-1 homolog A, gamma-secretase subunit
APOE	apolipoprotein E
APP	amyloid precursor protein
ASCL1	Achaete-Scute family BHLH transcription factor 1
BACE1	beta-secretase 1
BCL2L11	BCL2 like 11 protein
BDNF	brain-derived neurotrophic factor
bFCN	basal forebrain cholinergic neuron
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
CDK	cyclin-dependent protein kinase
CDX2	caudal type homeobox 2
ChAT	choline acetyltransferase
CLDN11	claudin 11
CLIC1	chloride intracellular channel 1
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CSNK2	casein kinase 2
CTF α	C-terminal fragment α
CTF β	C-terminal fragment β
DA	dopaminergic neuron
DAZL	deleted in azoospermia like
DKK1	Dickkopf WNT signaling pathway inhibitor 1
DLG4	discs large MAGUK scaffold protein 4
DLX	distal-less homeobox
DMR	differentially methylated region
DNM1L	dynamamin 1 like
DS	Down's syndrome
EB	embryoid body
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGR2	early growth response 2
EMX	empty spiracles homeobox
EN	engrailed homeobox
EpiSC	epiblast stem cell
ER	endoplasmic reticulum
ERBB3	Erb-B2 receptor tyrosine kinase 3
ERK	extracellular signal-regulated kinase

ESC	embryonic stem cell
ESR2	estrogen receptor 2
fAD	familial Alzheimer's disease
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FIS1	fission, mitochondrial 1
FOXP1	forkhead box G1
FP	floor plate
FTD	frontotemporal dementia
FUS	FUS RNA binding protein
FYN	FYN proto-oncogene, Src family tyrosine kinase
GABA	gamma-aminobutyric acid
GAD	glutamate acid decarboxylase
GAP43	growth associated protein 43
GATA4	GATA binding protein 4
GBA	glucosylceramidase beta
GBX	gastrulation brain homeobox
GDF2	growth differentiation factor 2
GDNF	glial cell derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFRA3	GDNF family receptor alpha 3
GLI3	GLI family zinc finger 3
GRIN1	glutamate ionotropic receptor NMDA type subunit 1
GRN	granulin precursor
GSH	glutathione
GSK3B	glycogen synthase kinase 3 beta
HD	Huntington's disease
hESC	human embryonic stem cell
HSC	hematopoietic stem cell
HSP	heat shock protein family
HTT	huntingtin
ICM	inner cell mass
IGF	insulin like growth factor
IL	interleukin
iPSC	induced pluripotent stem cell
IRX3	iroquois homeobox 3
ISL1	ISL LIM homeobox 1
JAK	Janus kinase
KLF4	Kruppel like factor 4
KSR	knock-out serum replacement
LHX8	LIM homeobox 8
LIF	leukemia inhibitory factor
LMX	LIM homeobox transcription factor
LOAD	late onset Alzheimer's disease
LRRK2	leucine rich repeat kinase 2
mAChR	muscarinic acetylcholine receptor
MAPK	mitogen-activated protein kinase
MAPT	microtubule associated protein TAU
MARK	microtubule affinity-regulating kinase
mDA	midbrain dopaminergic neuron
MECP2	methyl-CpG binding protein 2
MEF	mouse embryonic fibroblast
mESC	mouse embryonic stem cell
MFN	mitofusin
MGE	medial ganglionic eminence
MN	motor neuron

MX1	motor neuron and pancreas homeobox 1
mPTP	mitochondrial permeability transition pore
MSC	mesenchymal stem cell
MTBR	microtubule binding repeats
MYC	V-Myc avian myelocytomatosis viral oncogene homolog
nAChR	nicotinic acetylcholine receptor
NADPH	nicotinamide adenine dinucleotide phosphate
NANOG	Nanog homeobox
NEAA	nonessential amino acids
NES	nestin
NEUROG2	neurogenin 2
NF200	neurofilament, heavy polypeptide 200kDa
NF-L	neurofilament L
NFT	neurofibrillary tangle
NGF	neuronal growth factor
NGS	next generation sequencing
NKX2-2	NK2 homeobox 2
NKX6-1	NK6 homeobox 1
NMDAR	N-methyl-D-aspartate receptor
NO	nitric oxide
NOX	NADPH oxidase
NPC	neural progenitor cell
NR4A2	nuclear receptor subfamily 4 group A member 2
NROB1	nuclear receptor subfamily 0 group B member 1
NSC	neural stem cells
NT	neurotrophin
NT3	neurotrophin 3
OCT4	octamer-binding transcription factor4
OLIG2	oligodendrocyte lineage transcription factor 2
OPA1	OPA1 mitochondrial dynamin like GTPase
OTX	orthodenticle homeobox
P75NTR	neurotrophin receptor p75
PARK2	Parkin RBR E3 ubiquitin protein ligase
PAX	paired box
PD	Parkinson's disease
PDPK	3-phosphoinositide dependent protein kinase
PECAM1	platelet and endothelial cell adhesion molecule 1
PFA	paraformaldehyde
PHF	paired helical filament
PI3K	phosphoinositide 3-kinase
PINK1	PTEN induced putative kinase 1
PITX3	paired like homeodomain 3
PIWIL2	Piwi like RNA-mediated gene silencing 2
PLCG1	phospholipase C gamma 1
pMN	motor neuron progenitor
PNS	peripheral nervous system
POL/L	poly-L-ornithine/laminin
POU5F1	POU domain, class 5, transcription factor 1
PrPc	cellular prion protein
PRKAA1	protein kinase AMP-activated catalytic subunit alpha 2
PSC	pluripotent stem cell
PSEN	presenilin
PTP	permeability transition pore
qRT-PCR	quantitative real-time polymerase chain reaction
RA	retinoic acid
rbEF	rabbit embryonic fibroblast
rbESC	rabbit embryonic stem cell

RBFOX3	RNA Binding Protein, Fox-1 Homolog 3
rbiPSC	rabbit induced pluripotent stem cell
REXO1	RNA exonuclease 1 homolog
ROS	reactive oxygen species
RTT	Rett syndrome
SCID	severe combined immunodeficiency
SEMA3B	semaphorin 3B
SH3	Src-homology 3
SHH	sonic hedgehog
SIRT1	sirtuin 1
SLC6A4	solute carrier family 6 member 4
S.M.Actin	smooth muscle actin
SNCA	synuclein alpha
SOD1	superoxide dismutase 1
SOX2	sex determining region Y-box 2
SRC	SRC proto-oncogene, non-receptor tyrosine kinase
SSEA	stage-specific embryonic antigen
STAT3	signal transducer and activator of transcription-3
TARDBP	TAR DNA binding protein
TBR	T-box, brain
TBX3	T-box 3
TD	terminal differentiation
TGFB	transforming growth factor beta
TH	tyrosine hydroxylase
TNF	tumor necrosis factor
TP53	tumor protein p53
TUBB3	tubulin beta 3 class III
UCHL1	ubiquitin C-terminal hydrolase L1
UTF1	undifferentiated embryonic cell transcription factor 1
VACHT	vesicular acetylcholine transporter
VAPB	VAMP associated protein B and C
VGLUT1/2	vesicular glutamate transporter 1/2
VZ	ventricular zone

1. INTRODUCTION

1.1 Importance of the field

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. Due to their similarity to humans in terms of physiology, anatomy and genetics, as well as their unlimited supply, they are frequently selected for experimental disease research. 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 (Danielle, 2007). Among them, laboratory mice are the most routinely used mammals to study genetic diseases, because of the well-established culture and genome modification protocols, ease of handling and similarities in the genome to that of humans. 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 (Dunnett and Lelos, 2010), cell death in stroke (Hoyte *et al.*, 2010) or optic nerve injury (Wood *et al.*, 2011). 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. Neural stem cells 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.

1.2 Disease modelling with animals

Despite many similarities between human and animal genome, model organisms mostly do not reproduce the same genetic or phenotypic mechanisms. Often, animal genome must be altered to induce human disease phenotypes. If the genetic background of the disease is well known, the same mutations which cause disease in human, can be introduced into the corresponding animal gene.

Within all neurological disorders, neurodegenerative diseases such as Alzheimer's disease (AD) affect the highest percentage of population. Most available *in vivo* models of AD are made in mice. Due to the complexity of the disease, one single animal can present only one or two aspects of the disease spectrum. For example, the accumulation of amyloid β ($A\beta$) plaque and overexpression of microtubule associated protein TAU (MAPT) forming intracellular neurofibrillary tangles (NFT), the two pathological hallmarks of AD, were a common target in generation of animal models (Cavanaugh *et al.*, 2014). Numerous transgenic mouse models were generated with mutations in amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) genes, associated with familial AD (fAD). In most *APP* mutants, extracellular deposits of $A\beta$ were secreted at different time points. Moreover, mutant animals displayed astrocytosis, microgliosis, neurotransmission disturbances, and cognitive and behavioural deficits (Van Dam *et al.*, 2011). Mice expressing mutated form of human *PSEN1* or *PSEN2* alone have not produced $A\beta$ plaques, whereas double *APP/PSEN1* mutants developed $A\beta$ deposits in a much earlier age (Casas *et al.*, 2004). *APP* mutants exhibited neuronal loss and TAU hyperphosphorylation in some regions of the brain, although NFT formation was not observed (Götz *et al.*, 2007). To model the NFT, transgenic mice with mutations in human *APP* and *TAU* were generated. These animals developed NFTs and helped to clarify the relationship between TAU and $A\beta$ (Lewis, 2001). Use of transgenic mouse models of amyloid pathology provides a new insight into the processing of $A\beta$, for instance, functional and pathological changes in AD models occur before the $A\beta$ plaque formation (Schaeffer *et al.*, 2011). Although AD mouse models recapitulate many aspects of the disease, so far no drug has been developed successfully using these *in vivo* models. The limitations are related to the absence of the extensive neuronal cell loss in AD models and NFT formation in most *APP* models. Only triple transgenic mouse (mutant *APP*, *PSEN1* and *TAU*) developed $A\beta$ deposits, NFT, together with inflammation, synaptic dysfunction and cognitive decline (Oddo *et al.*, 2003).

In contrary to mice, rabbits have the same $A\beta$ peptide-sequence as humans that prevents spontaneous development of any AD-like disease (Johnstone *et al.*, 1991). Additionally, rabbits

are closer relatives with primates than rodents. In a study on wild type rabbit feeding, a high cholesterol diet induced development of A β plaques and TAU pathology, as well as neuronal loss and cognitive impairment (Sparks and Schreurs, 2003). Moreover, the level of A β depositions was dependent on the presence of copper in the drinking water. It was speculated that this metal may be involved in AD progression (Woodruff-Pak *et al.*, 2011). Thus, some studies have been performed to test metal chelators as a potential drugs in neuroprotective therapy (Woodruff-Pak *et al.*, 2011).

Although animal models are involved in study of AD pathogenesis, they do not exhibit all AD features. Currently, most of the animal models are generated based on known genetic mutations. However, the majority of AD cases are sporadic and their genetic background is not known. As a result, the animal models do not recapitulate all features of sporadic AD and do not cover all aspects that can lead to the etiopathogenesis of sporadic AD. Thus, reducing any one of the different risk factors contributing to AD progression by improving the neural environment (vitamins, antioxidants, vasodilators, etc.) may significantly improve the incipient AD-phenotype in animals.

1.3 Disease modelling *in vitro*

While animal disease models have the potential to advance the study of neurological disorders, many of these models are inefficient for faithful recapitulation of the human conditions. Additionally, identification of critical cellular and molecular processes contributing to disease or their independent modification in a whole animal model is very difficult. The ability to model human disease *in vitro* using pluripotent stem cells (PSCs) has changed this field. The availability of pluripotent stem cell-based disease modelling is a good alternative to invasive or often unfeasible brain or spinal cord biopsies. Additionally, the development of induced PSC (iPSCs) technology by Yamanaka's group (Takahashi *et al.*, 2007) made possible to generate pluripotent stem cells from the somatic cells of any individual, including patients carrying specific mutations and genetic risk factors. Self-renewal capability of PSCs and their potential to differentiate into any cell type (including specific cell types relevant for a given disorder), allow to overcome the limitations of animal models for certain neurological diseases. Mechanisms contributing to central nervous system neuropathology can be investigated by differentiating PSCs towards particular neural subtypes or glia cells in a dish and studying the relevant cell populations affected in the given disease in terms of specific cellular phenotype. So far, the most efficient generation of specific neuronal cells was established using dual inhibition of SMAD

signalling in a feeder-free culture system. NOGGIN (inhibitor of bone morphogenetic protein - BMP) and SB431542 (inhibitor of transforming growth factor beta - TGFB) rapidly and efficaciously generated neuroepithelial cells, which had a potential to differentiate into various region-specific neurons using appropriate factors (Chambers *et al.*, 2009a). Parallel to the generation of new stem cell lines, the identification of stem cell niches within most adult tissues was carried out. Maintenance of stem cells within a tissue in an adequate environment provides a source of cells, which may be used to model disease conditions.

Most available *in vitro* models of AD are based on embryonic stem cells (ESCs) and iPSCs. Recent work reported that the iPSC-derived neurons from patients with mutations in *APP*, *PSEN1* or *PSEN2* gene exhibited increased A β expression, TAU hyperphosphorylation and activation of glycogen synthase kinase 3 beta (GSK3B) (Yagi *et al.*, 2011; Israel *et al.*, 2012). Neurons derived from patients with sporadic AD (sAD) also revealed higher accumulation of A β (Israel *et al.*, 2012). Inhibition of γ -secretase, an intra membrane protease complex containing PSEN1 and PSEN2 decreased A β production in neurons derived from *PSEN1* and *PSEN2* mutants. The same effect was observed when sporadic AD and *APP* mutant neurons were cultured with β -secretase inhibitor, but not the γ -secretase inhibitor (Yagi *et al.*, 2011; Israel *et al.*, 2012). Despite the above achievements, a selective loss of cortical neurons and impaired synaptic functions, the main pathological changes in AD, were not detectable. It can be the effect of immature neuronal culture with not fully developed disease phenotype or the absence of appropriate stressors related to ageing. Interestingly, later studies have shown that neuronal loss in AD can be modelled by incubation of forebrain cholinergic neurons derived from iPSCs with A β 1-42 aggregates (Xu *et al.*, 2013).

These observations reveal that stem cells can offer innovative approaches to study disease mechanisms. The iPSCs allows elucidating neurological disorder phenomena and testing various clinical therapies. Furthermore, *in vitro* models give a possibility to design novel and effective drugs that may lead to development of new strategies for the treatment of genetic and sporadic diseases.

1.4 Objectives

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
- Characterisation 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. OVERVIEW OF THE LITERATURE

2.1 Stem cells

2.1.1 Stem cells and their unique properties

Stem cells are certain biological cells in small portion in the body that have remarkable potential to differentiate into diverse specialized cell types during life and growth. Stem cells can divide asymmetrically to give rise to two distinct daughter cells, one copy of the original stem cell and one cell programmed to differentiate into a non-stem cell fate. During a development or regeneration stem cells can also divide symmetrically to produce two identical copies of the original cell. There are three unique properties of all stem cells regardless of their source. These include: capability of dividing and renewing themselves for long periods, being unspecialized and basic cells, and potential to differentiate into any type of specialized cell. Different types of stem cells vary in their degree of plasticity or developmental versatility. Stem cells can be classified according to their plasticity and origin. The capacity of stem cells to differentiate into various cell types can be defined by their potency:

- totipotent stem cells – the cells with the capacity to self-renew and develop into the three germ layers and extraembryonic tissues such as placenta; they have potential to give rise to an entire functional organism
- pluripotent stem cells – the cells are the descendants of totipotent cells and can differentiate into nearly all cells excluding extraembryonic tissues
- multipotent stem cells – the progenitor cells, which are able to differentiate into a limited range of cells within a germ layers
- unipotent stem cells – the cells with very limited differentiation potential, they can differentiate into only one type of cell or tissue, self-renewal property distinguishes them from non-stem cells

Stem cells are found in preimplantation stage embryos, fetuses, in the umbilical cord, and in many tissues of the fully developed body, most notably in the bone marrow. Stem cells may be classified according to their origin into three major groups: embryonic, fetal and adult stem cells.

2.1.2 Embryonic stem cells

ESCs are derived from the inner cell mass (ICM) of a blastocyst or from embryos at the morula stage. During development, ESCs are able to differentiate into all derivatives of the three germ layers: ectoderm, endoderm and mesoderm. Following sufficient and necessary stimulation for a specific cell type, ESCs can develop into more than 200 cell types of the adult body (Hui *et al.*,

2011). Pluripotent stem cells can exist in two morphologically, molecularly and functionally distinct pluripotent states: naive and primed. Naive ESCs have unlimited self-renewal capacity and are able to differentiate into all three germ layers *in vitro*. After injecting them into the blastocoel or aggregating them with early preimplantation stage embryos they give rise to all somatic lineages including the germline, and are able to form chimeras (Gardner, 1968; Nagy *et al.*, 1990). Primed ESCs present also self-renewal potential and contribute to all three germ layers *in vitro*, however they are not able to give rise to the germline chimeras *in vivo*, which was confirmed in mice (Huang *et al.*, 2012). Due to an unlimited capacity for self-renewal, ESCs have been proposed as a new method in regenerative medicine or tissue engineering for replacement therapy as reviewed in (Zhang, 2003). Potentially ESCs can be used for treatment of many diseases including: blood and immune system related genetic diseases, neurological disease or cancers as reviewed in (Ukrainitseva and Yashin, 2005; Lindvall and Kokaia, 2006).

2.1.3 Fetal stem cells

Fetal stem cells are primitive cell types found in fetal blood, bone marrow and other fetal tissues and organs such as liver, kidney, pancreas and neural crest. Fetal blood is a rich source of hematopoietic stem cells (HSCs) with very high proliferation rate, non-hematopoietic mesenchymal stem cells (MSCs), which support blood cell formation and can differentiate into multiple lineages and cord blood stem cells that can be used to generate red blood cells and cells of the immune system. Fetal liver stem cells isolated from human fetus have shown enormous proliferation and after transplantation into animals they differentiated into mature hepatocytes (Soto-Gutierrez *et al.*, 2009). Thus, fetal liver stem cells may be a suitable alternative to overcome the limitations of liver engraftments and to allow a functional correction of the disease phenotype (Khan *et al.*, 2010). Fetal neural stem cells isolated from fetal brain have shown differentiation capacity to produce neurons and glial cells (Villa *et al.*, 2000). In terms of downstream application, fetal stem cells are less ethically contentious than ESCs and their differentiation potential is higher than adult stem cells. Fetal stem cells with great multipotentiality and low immunogenicity are promising tool for cell transplantation and gene therapy. Thus, to provide a resource of stem cells for medical research and clinical application, umbilical cord blood samples are collected and stored either in public or private cord blood banks. These stem cells are used mostly in the treatment of children, but have been also applied in adults following chemotherapy treatment. Currently their major application is in the treatment of blood and immune system disorders such as leukemia, anemia and autoimmune disease (Ishii and Eto, 2014).

2.1.4 Adult stem cells

Adult stem cells known as somatic stem cells are rare and generally small in number. They can be found in mature tissues and they are generally referred to by their tissue origin, e.g. adipose derived stem cells, pancreatic stem cells, mesenchymal stem cells (Barrilleaux *et al.*, 2006). Because of the stage of development, they have limited potential compare to ESCs. Most of them are lineage-restricted (multipotent) with the ability to divide and generate all cell types of the organ from which they originate. Adult stem cells play important role in local tissue repair and regeneration. Their application in research and therapy is not as controversial as ESCs because the generation of these cells does not require the destruction of a human embryo at any stage. Furthermore, adult stem cells can be obtained from the intended recipient as an autograft that completely eliminate the risk of tissue rejection. So far adult stem cell were applied in treatment of leukemia and related bone/blood cancers (Lown *et al.*, 2014), spinal cord injury (Srivastava *et al.*, 2010), liver cirrhosis (Terai *et al.*, 2006). There are also applications reported in the field of veterinary medicine (Marx *et al.*, 2014).

2.1.5 Stem cell regulatory pathways

Pluripotency and self-renewal are two crucial hallmarks of human embryonic stem cells. This state is controlled by a transcriptional regulatory network and signaling pathways. Critical components involved in the maintenance of the stem cell pluripotency include transcription factors, signaling molecules, chromatin regulators, histone modifications and regulatory RNAs. The most important signaling pathways that allow pluripotent stem cells divide continuously in the undifferentiated state are TGF β /ACTIVIN/NODAL which act through SMAD 2/3/4, insulin/insulin like factor (IGF) which acts through phosphoinositide 3-kinase (PI3K) and fibroblast growth factor receptor (FGFR) that activates the mitogen-activated protein kinase (MAPK) and AKT serine/threonine kinase (AKT) pathways. The role of canonical WNT pathway in the maintenance of pluripotency is controversial. Some groups has shown that WNT promotes pluripotency by non-canonical mechanism involving a balance between the transcriptional activator TCF1 and the repressor TCF3 (Yi *et al.*, 2011). However the recent studies indicate that WNT signaling promotes differentiation by repressing genes that stimulate self-renewal (Davidson *et al.*, 2012). Signaling through TGF β , IGF and FGFR results in activation of three essential pluripotency associated genes: POUclass 5 homeobox 1 (*POU5F1*) also known as octamer-binding transcription factor 4 (*OCT4*), sex determining region Y-box 2 (*SOX2*) and Nanog homeobox (*NANOG*). These transcription factors are considered to form a self-sustaining gene regulatory network involved in the suppression of differentiation and the maintenance of the pluripotency (Niwa, 2007). OCT4 and NANOG based on their unique

expression pattern in ESCs were identified as a key pluripotency regulators, essential for maintenance of a robust pluripotent state (Chambers *et al.*, 2003). SOX2 which acts as a heterodimer with OCT4, play also critical role among the key regulators (Masui *et al.*, 2007). NANOG promotes a stable undifferentiated ESC state and is important for pluripotency of ICM cells (Pan and Thomson, 2007). Furthermore, NANOG co-occupies most sites with OCT4 and SOX2 throughout ESC genome and suppresses differentiation of PSCs toward the extraembryonic endoderm and trophectoderm lineages (Marson *et al.*, 2008). The core transcription factors bind together at their own promoters and often co-occupy their target genes, forming an interconnected autoregulatory loop. These factors activate expression of the pluripotency genes but also contribute to repression of genes encoding cell lineage-specific transcription factors. The loss of the core regulators causes a rapid activation of wide spectrum of genes encoding lineage specific regulators and differentiation of PSCs (Young, 2011). Recent studies have shown that NANOG can interact with BMP signaling pathway and adversely affect the pluripotency state. BMPs belong to the TGF β superfamily and mediate signaling through SMAD1/5/8 that activate the expression of differentiation-specific genes. BMPs promote the expression of T Brachyury transcription factor (T) – a mesoderm marker and prevent the neuroectoderm differentiation (Finley *et al.*, 1999). In the presence of leukemia inhibitory factor (LIF), the activated form of signal transducer and activator of transcription 3 (STAT3) binds the NANOG promoter and upregulate its expression (Suzuki *et al.*, 2006). High level of NANOG can interact with SMAD1 and interfere with the further recruitment of the coactivators for the active SMAD1 complexes that leads to inhibition of BMP activity (Suzuki *et al.*, 2006). Consequently, mesoderm progression is limited and ultimately the undifferentiated state of ESCs is maintained.

2.2 Human stem cells

2.2.1 Human embryonic stem cells

Almost two decades after the isolation of the first ESC line, the first human ESCs (hESCs) were established in 1998 by Thomson *et al.* from *in vitro* human blastocysts (**Figure 1A**). Despite these achievements derivation of ESCs from other species such as farm animals was inefficient, probably due to very limited knowledge about isolation and maintenance of the different species preimplantation stage embryos and ESCs. Although hESCs show the essential stem cell characteristics, they require special culture conditions to maintain an undifferentiated state. These cells were originally cultured on an inactivated mouse embryonic fibroblasts (MEFs)

feeder layer in medium supplemented with fibroblast growth factor 2 (FGF2) and bovine serum (Thomson, 1998). Then, a feeder-free systems have been developed, in which hESCs grown on a protein matrix (Matrigel or Laminin) in the presence of FGF2 in medium, which was previously conditioned by co-culture with fibroblasts (Xu *et al.*, 2001). Nowadays highly specialized, serum-free and complete cell culture medium are used to optimize the hESC proliferation and reduce their potential for differentiation (Zhang *et al.*, 2016).

The hESCs are characterized by well-established criteria including the capacity to differentiate into all somatic cell types of the body *in vitro* and teratoma formation *in vivo*. Under defined conditions hESCs can differentiate spontaneously in three-dimensional aggregates and form embryoid bodies (EBs). Within EBs hESCs form cell-cell contacts and differentiate into all cell types of the three germ layers and display some common features of pregastrulation and early gastrulation (Weitzer, 2006). Gene expression studies in ESCs have revealed many proteins involved in the ‘stemness’ phenotype that can function as endogenous ESC markers such as OCT4, SOX2 and NANOG. The most commonly used markers for hESCs identification are cell surface antigens: stage-specific embryonic antigen 3 and 4 (SSEA-3, SSEA-4) and the keratan sulfate antigens TRA-1-60 and TRA-1-81 (Hui *et al.*, 2011). The potential to generate any type of cells from ESCs gives a possibility to obtain large numbers of cells for cell therapy or regenerative medicine. However, due to the difficulties in controlling of proliferation and differential potential, the application of ESCs *in vitro* are currently limited. Some studies revealed that prolonged culture of hESCs increased the potential for accumulation of genetic, epigenetic and karyotypic changes (Draper *et al.*, 2004; Inzunza *et al.*, 2004). Additionally, manipulations with human embryos have ignited controversy over the using hESCs in stem cell research and medicine.

2.2.2 Human induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are a type of pluripotent stem cells that can be generated directly from differentiated somatic adult cells by genetic reprogramming (**Figure 1B**). The first successful reprogramming of MEFs into iPSCs by retroviral transduction was established by Yamanaka’s group (Takahashi and Yamanaka, 2006). This finding defined a combination of four transcription factors: OCT4, SOX2, Kruppel like factor 4 (KLF4) and V-Myc avian myelocytomatosis viral oncogene homolog (MYC) known as cellular-myelocytomatosis oncogene (c-MYC) as both necessary and sufficient molecules to convert terminally differentiated cells into PSCs upon their forced expression (overexpression) in differentiated cells. The generated iPSCs are capable to differentiate into all cell types of the three germ layers, similar to the capacity of ESCs. Since then, iPSC technology evolved into productive and fast developing

research field. Many laboratories derived iPSCs from various donor cell sources, such as neuronal cells (Dimos *et al.*, 2008), keratinocytes obtained from a single hair pluck (Aasen *et al.*, 2008), adipose stromal cells (Sun *et al.*, 2009), peripheral blood cells (Staerk *et al.*, 2010), renal epithelial cells in the urine (Zhou *et al.*, 2012) and others. Reprogramming of adult cells into iPSCs using viral transduction may pose significant risks that could limit their use in humans. Genomic integration of the transcription factors into the target cell genome may cause mutations, additionally the expression of oncogenes such as c-MYC may be potentially triggered. A common strategy to avoid genomic insertion or tumor genesis has been to use a different vector for input: adenovirus, plasmids, transposon vectors or protein compounds. In 2008, Stadtfeld *et al.* demonstrated successful reprogramming of mouse skin and liver cells using an adenovirus system for delivery the requisite four transcription factors into the donor cells (Stadtfeld *et al.*, 2008). One year later similar experiment was performed during reprogramming human fibroblast into iPSCs (Zhou and Freed, 2009). The advantages of adenoviruses are that, in contrast to retroviruses they do not incorporate their own genes into the targeted cells and avoid the potential for insertional mutagenesis. Additionally, only a brief amount of time of adenovirus presence in the host is required to effectively reprogram the cells. In 2008, Okita *et al.* reprogrammed mouse cells by transfection with two plasmid constructs carrying *c-Myc* on the first plasmid and *Oct4*, *Sox2* and *Klf4* on the second plasmids (Okita *et al.*, 2008). However, this method seemed to be less efficient compared to retroviral methods, and still require cancer – promoting genes for reprogramming. Furthermore, transfected plasmids integrated into the host genome that increased the risk of mutagenesis. Due to low efficiency level of non-retroviral approaches, a new delivery method based on transposon system was tested. Several studies demonstrated that PiggyBac transposon system can effectively deliver transcription factors into the genome without leaving footprint mutations (Woltjen *et al.*, 2011). The above transposon contain the re-excision of exogenous genes that entirely eliminates insertional mutagenesis. At the same time few research groups in line with ours have shown that generation of iPSCs is possible without any genetic alteration of the adult cell by a treatment of the cells with certain proteins (Zhang *et al.*, 2012; Nemes *et al.*, 2014). So far many researchers established iPSCs with nearly identical functionality to ESCs. Because iPSCs are derived directly from somatic cells there is no ethical controversies related to the destruction of human embryos or oocytes. Furthermore, iPSCs provide an unlimited source of proliferating cells, that hold a great promise for regenerative medicine as reviewed in (Singh *et al.*, 2015).

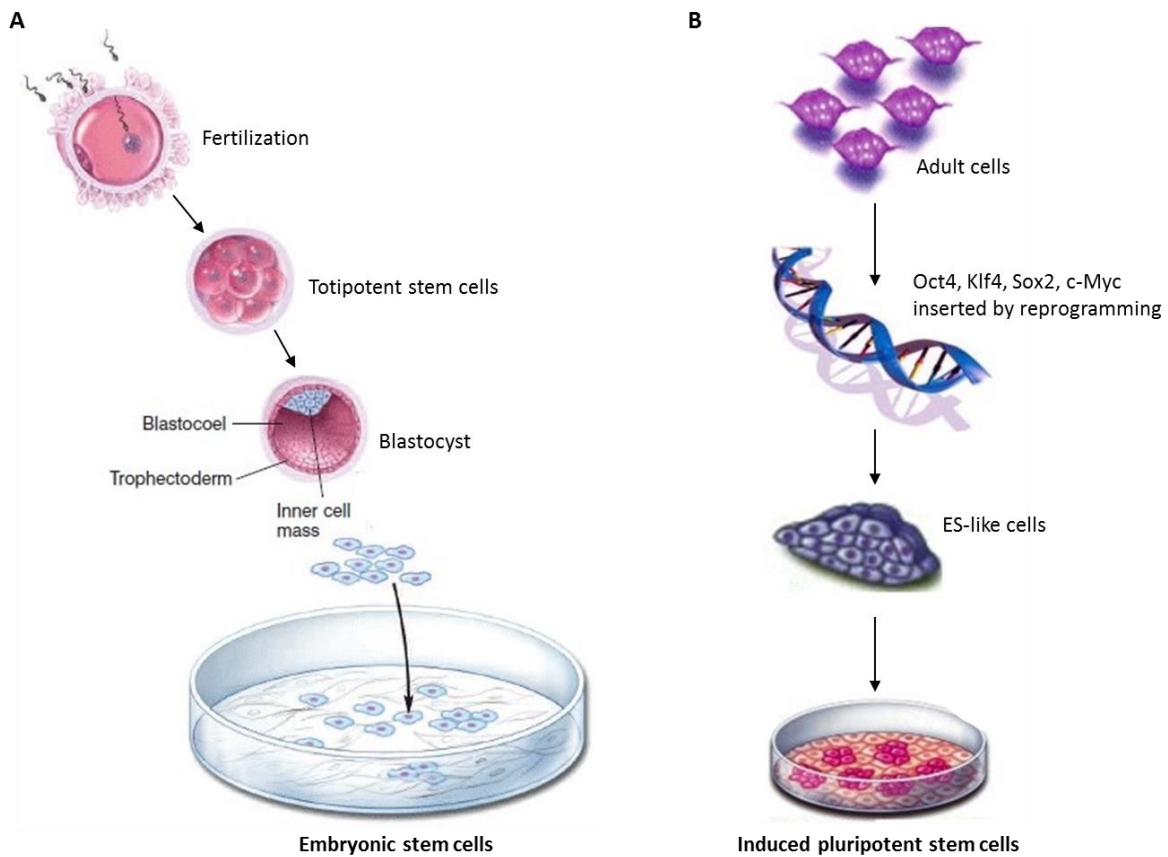


Figure 1. Pluripotent stem cells. (A) Generation of embryonic stem cells (ESCs) from inner cell mass (B) and induced pluripotent stem cells (iPSCs) by reprogramming of adult cells with OCT4, SOX2, KLF4 and c-MYC transcription factors. (Adapted from Winslow, 2001).

2.2.3 Comparison of human ESCs and iPSCs

Human iPSCs derived from a non-pluripotent cell by inducing overexpression of specific “pluripotency” genes are similar to natural pluripotent stem cells such as ESCs in many aspects (e.g. the expression of certain genes and proteins, embryoid body formation, teratoma formation), but all their relations to ESCs are still not well examined (Kingham and Oreffo, 2013). The recent studies have revealed substantial differences in genetic and epigenetic profiles of iPSCs vs ESCs and their differentiation potential as reviewed previously (Bilic *et al.*, 2012). Chin *et al.* reported that the transcription profiles of iPSCs and ESCs are nearly identical, however there is a small group of genes which is continuously differentially expressed in iPSC and ESC lines (Chin *et al.*, 2009). In contrast to this statement, two other groups using different statistical algorithms have found some differences between iPSC and ESC expression profile, but they were caused rather by different laboratory culture conditions than various pluripotency state (Guenther *et al.*, 2010; Newman and Cooper, 2010). It can suggest that iPSCs and ESCs belong to the same class of pluripotent stem cells in their gene expression signature. However, the

analysis of miRNA expression profile, especially tumor protein p53 (TP53) network has shown that iPSCs overexpressed the p53-targeting miRNAs: miR-92 and miR-141. The miR-92 regulates primed pluripotent stem cell survival through targeting the pro-apoptotic BCL2 like 11 protein (BCL2L11) (Pernaute *et al.*, 2014). These findings suggested a subdivision of pluripotent stem cells into two distinct categories independent of their origin but related to TP53 network status (Neveu *et al.*, 2010). Additionally, it was reported that p53 is regulated by sirtuin 1 (SIRT1), which promotes cell survival via inhibiting programmed cell death and maintains the naïve state of embryonic stem cells (Williams *et al.*, 2016).

The development of high-throughput sequencing technologies and generation of single-nucleotide genome-wide maps of DNA methylation gave an insight into epigenetic differences between iPSCs and ESCs. The recent studies revealed very similar DNA methylation pattern in iPSCs and ESCs. The analysis of the whole genome of three iPSC lines and three ESC lines identified 71 differentially methylated regions (DMR), in which almost half of the DMRs show incomplete epigenetic reprogramming of the differentiated cell-of-origin genome (Doi *et al.*, 2009). Not all recognized DMRs belong to the cell-of-origin memory, that indicates accumulation of novel aberrant epigenetic modifications in iPSCs (Lister *et al.*, 2011).

Similar to ESCs, both hypermethylated and hypomethylated CpG sites were found in iPSCs (Lister *et al.*, 2011). However, hypomethylation of CpG have increased in iPSCs, that can suggest there is an inefficient methylation during the reprogramming. These aberrations in the CpG methylation are not transient and they are detected in high passage number of iPSCs. During reprogramming iPSCs regain non-CpG methylation, which is characteristic for ESCs (Lister *et al.*, 2011). Differentiation of iPSCs into trophoblast cells revealed that perturbations in CpG methylation are transmitted at a high frequency and maintained after differentiation, providing characteristic iPSC reprogramming signature.

2.3 Rabbit stem cells

2.3.1 Rabbit embryonic stem cells

ESC lines have been successfully established in many animal species like monkeys, rats and mice (Klimanskaya *et al.*, 2013). The first rabbit ESCs (rbESCs) were derived from blastocysts in their preimplantation stage (Graves and Moreadith, 1993). These cells exhibited the cardinal features of pluripotent stem cells: the ability to grow in undifferentiated states and capacity to form terminally differentiated cell types representative of ectoderm, mesoderm and endoderm.

Similar to human and mouse ESCs (mESCs), rbESCs expressed the pluripotency genes *OCT4*, *NANOG*, *SOX2*, and undifferentiated embryonic cell transcription factor 1 (*UTF1*) as well as alkaline phosphatase (AP), stage specific embryonic markers (SSEA-1, SSEA-2, SSEA-4) and the tumor related antigens (TRA 1-60 and TRA 1-81) (Wang *et al.*, 2007). Morphologically rbESCs resembled primate human ESCs, they showed high nucleus/cytoplasm ratio, prominent nucleoli, and distinct cell borders, which formed flat cell colonies. One of the critical factor for derivation and culture of rbESCs appeared to be the feeder cell density. It was found that feeder cell density determines the fate of rbESCs and the maximum proliferation potential was reached when ESCs were cultured on MEFs at a concentration of $3,6 \times 10^4$ cells/cm² (Honda *et al.*, 2008). Higher and lower feeder cell densities repressed proliferation or induced ESC differentiation. This suggests that MEFs, which are mostly used as the feeder layer cells, inhibit proliferation of rbESCs by competing for surface area or through a contact mediated mechanism. Under optimized culture conditions rbESCs displayed unlimited growth (until passage 50) and high telomerase activity (Honda *et al.*, 2008). Spontaneous differentiation into fibroblast-like cells and cell death occurred rapidly when rbESCs were cultured in the absence of feeder cells (Wang *et al.*, 2007). This finding indicated the indispensability of feeder cells for the maintenance of the rbESC pluripotency. Previous studies revealed several signal transduction pathways involved in the maintenance of the ESC self-renewal including FGF, TGF/BMP and WNT pathways (Brandenberger *et al.*, 2004; Sato *et al.*, 2004; Xu *et al.*, 2005). The rbESCs expressed several FGF signaling pathways genes and also the components of downstream activation cascade (Wang *et al.*, 2007). The expression of FGF genes may suggest that rbESCs require FGF signaling pathway to maintain the undifferentiated state. It has been shown that activation of extracellular signal-regulated kinase (ERK) and PI3K by FGF2 are necessary to preserve cell pluripotency (Wang *et al.*, 2008). FGF2 together with LIF cooperatively support self-renewal in rbESCs derived from parthenogenetic blastocysts and propagated in feeder free conditions (Wang *et al.*, 2007). However, studies performed by Honda *et al.* demonstrated that withdrawal of LIF and inhibition of a Janus kinase (JAK) resulting in the loss of phosphorylated signal transducer and activator of STAT3 in the presence of feeder cells, was dispensable for self-renewal of rbESCs (Honda *et al.*, 2008; Osteil *et al.*, 2013). Unlike ESCs generated in mice and humans, rbESCs exhibit G1 growth arrest after DNA damage, revealing the presence of a checkpoint before entry into S phase of cell cycle similar to somatic cells (Osteil *et al.*, 2013).

2.3.2 Rabbit induced pluripotent stem cells

Human iPSCs have many potential applications to regenerative medicine and transplantations. Therefore, the safety and the efficiency of iPS-derived cells must be verified using appropriate animal models before human clinical trials can start. Moreover, animal species, like rabbit has more advantages in disease modelling as well as in animal husbandry as an important farm animal. Thus the existence of iPSCs could increase the potential of rabbit as a model animal, by providing a stem cell source for genome manipulations as well (Bosze *et al.*, 2003; Osteil *et al.*, 2013). The first rabbit iPSCs (rbiPSCs) were established by Honda *et al.* in 2010. Using lentiviral vectors carrying four human reprogramming factors *OCT4*, *SOX2*, *KLF4* and *c-MYC*, rabbit liver and stomach cells were successfully converted into pluripotent stem cells (Honda *et al.*, 2010). Newly generated rbiPSCs closely resembled human iPSCs, they grown in the presence of FGF2 and formed flattened colonies with sharp edges. The endogenous expression of the rabbit pluripotency markers such as *c-MYC*, *KLF4*, *SOX2*, *OCT3/4* and *NANOG* was detected, whereas the introduced human genes were completely silenced. Additionally, rbiPSCs have shown alkaline phosphatase activity and telomerase activity and they differentiated into the three germ layers. They also formed teratomas containing variety of tissues of all three germ layers but were unable to form chimeras *in vivo*. The global gene expression analysis revealed slight, but definite differences between rabbit ESCs and iPSCs. The same group attempted to reprogram also fetal and adult fibroblast from rabbits (Honda *et al.*, 2010). Fibroblasts are the most commonly used somatic cells for reprogramming in other species. It was reported that human iPSCs generated from fetal fibroblasts were more similar to ESCs in their global gene expression pattern than those from other cell types (Ghosh *et al.*, 2010). Despite these findings, rabbit fetal fibroblasts could not be successfully reprogrammed into PSCs by lentiviral transduction. That was probably caused by an exceptionally high proliferation rate of fetal rabbit fibroblasts *in vitro* which immediately reached confluence and discontinued dedifferentiation (Honda *et al.*, 2010). It can suggest that the initial proliferation rate of somatic cells is a defining factor for iPSC establishment in rabbits.

In 2013, Osteil *et al.* established three lines of rbiPSCs by reprogramming of adult skin fibroblasts from New Zealand White rabbits using four retroviral vectors carrying human *OCT4*, *SOX2*, *KLF4* and *c-MYC* transcription factors (Osteil *et al.*, 2013). All lines after injection into severe combined immunodeficiency (SCID) mice formed teratoma containing cells that differentiated into all three germ layers. The rbiPSCs have displayed characteristic features of naive pluripotency such as resistance to single-cell dissociation, high activity of the distal enhancer of the mouse *Oct4* gene, no expression of *N-cadherin* and expression of the rabbit ICM

markers. Successful conversion of rabbit fibroblasts into rbiPSCs could be the result of optimized culture conditions. Contrary to Honda's group, rbiPSCs were maintained in non-hypoxic conditions, in medium supplemented with higher concentration of FGF2, and in the absence of ESGRO (murine leukemia inhibitory factor). Despite high reprogramming efficiency, rbiPSCs did not express all the molecular markers of naive pluripotency including RNA exonuclease 1 homolog (REXO1), gastrulation brain homeobox 2 (GBX2), T-box 3 (TBX3) and nuclear receptor subfamily 0 group B member 1 (NROB1) (Tesar *et al.*, 2007). Furthermore, after injection into rabbit blastocysts, they showed a reduced capacity to colonize the ICM.

2.3.3 Comparison of rabbit ESCs and iPSCs

Despite the high similarity between rbESCs and rbiPSCs, there are several fundamental differences between these cells, which may have important implications regarding future applications in medicine and disease modelling. The recent studies have shown the significant differences in the maintenance conditions of rabbit PSCs. The rbESCs grow in flat colonies which can be passaged after collagenase II treatment followed by gentle dissociation into small clumps (Osteil *et al.*, 2013). Treatment of rbESCs with trypsin resulted in extensive cell death and differentiation (Tesar *et al.*, 2007). RbiPSCs similar to mESCs are resistant to trypsinization to single cells suspension. All pluripotent stem cells regardless of species express E-cadherin, a catenin complex involved in cellular adhesion, whereas another cell-cell adhesion molecule, N-cadherin is expressed only in mouse epiblast stem cells (EpiSCs) (Tesar *et al.*, 2007). The rbESCs and rbiPSCs displayed different pattern of E- and N-cadherin expression. All rbESCs express both E- and N-cadherin similar to mouse EpiSCs, while rbiPSCs expressed only E-cadherin (Osteil *et al.*, 2013). Cell cycle analysis has revealed that rbESCs had a longer G1 phase than the S and G2 phases, and comparable to somatic cells, they undergo growth arrest in the G1 phase after DNA damage. In contrast to the above data, rbiPSCs as well as mouse and primate PSCs have a relatively short G1 phase and lack of DNA damage checkpoint in the G1 phase. Instead, they undergo growth arrest only at the G2 checkpoint (Fluckiger *et al.*, 2006; Filipczyk *et al.*, 2007). Thus, rbESCs lack some key features of the pluripotent cell cycle, which might explain why they exhibit such a low proliferation rate and high spontaneous differentiation rate than rbiPSCs and primate ESCs (Osteil *et al.*, 2013). Further analysis has displayed a difference in the regulating of OCT4 expression. The distal element of OCT4 enhancer and its CR4 element, responsible for recruitment the OCT4 and SOX2 transcription factors, exhibited more robust activity in rbiPSCs than in rbESCs. Additionally, CR4 and CR1 elements of the OCT4 enhancer were less methylated in rbiPSCs than in ESCs (Osteil *et al.*, 2013). Gene expression analysis has detected 14 genes, which expression differs between rbESCs and rbiPSCs. In

rbESCs the expression of estrogen receptor 2 (*Esr2*), *Klf4*, Piwi like RNA-mediated gene silencing 2 (*Piwi12*), deleted in azoospermia like (*Dazl*) and platelet and endothelial cell adhesion molecule 1 (*Pecam1*) was higher compared to rbiPSCs. Based on the expression of 22 selected genes, rbiPSCs are more similar to rabbit ICM than rbESCs (Osteil *et al.*, 2013).

2.4 Using rabbit as an animal model

Rabbit (*Oryctolagus cuniculus*) is being used as an animal model in many branches of medical research. According to the United States Department of Agriculture, in 2015, rabbits were the second most commonly used animal in research in the USA. Among various strains, New Zealand White rabbits are the most commonly used strains in biomedical research (Mapara *et al.*, 2012). Compare to other breeds, these strains are less aggressive and have less health problem. One of the most important uses of rabbits is for antibody production, development of new surgical techniques and testing of new drugs and chemicals. Rabbits have many advantages and can fill the gap between small animal models, which are suitable for the first discovery phases of study and larger animals used for preclinical research. These animals are phylogenetically closer to primates than rodents, and have more diverse genetic background than inbred and outbred rodent strains (Graur *et al.*, 1996). Therefore, they mimic human genetic diversity more accurately, that make them more suitable for studying complex diseases such as atherosclerosis. Compare to large animal models, rabbits are relatively easy to handle, very economical and widely bred. Their vital cycles (gestation, lactation and puberty) are short and they are enough large to permit non-lethal monitoring of physiological changes. Furthermore, the key gene expression and functions are more close to humans. Additionally, rabbit genomics and proteomics are rapidly advancing fields that allowed to generate several transgenic lines. So far, transgenic rabbits have been used as a bioreactors for the production of pharmaceutical proteins, and animal models for a variety of human diseases (Houdebine, 1995). There are some human disorders, that cannot be modelled by rodents, thus, rabbits with their special anatomy and physiology can be appropriate for the study of these exact diseases (Bősze and Houdebine, 2006). The diseases, for which rabbits are used as a primary experimental model include tuberculosis, atherosclerosis, osteoarthritis and Alzheimer's disease. However, rabbits are also served as a model for the study of cardiovascular diseases, such as hypertrophic cardiomyopathy and lipid metabolism (Woodruff-Pak, 2008).

Nowadays rabbit model of hypercholesterolemia, originally created to study the atherosclerosis, is one of the leading model for investigation AD pathology (Woodruff-Pak *et al.*, 2011). Rabbits

fed high cholesterol diet exhibited many neuropathological changes at the cellular and molecular levels, which were observed in AD patients. Analysis of the rabbit brain revealed upregulation of A β and cholesterol levels, increased TAU and apolipoprotein E (APOE) expression, decreased acetylcholine (ACh) secretion, a blood-brain barrier breakdown, as well as a reduce in neurons and increase in microglia cell populations. Furthermore, rabbits with hypercholesterolemia have shown age dependent deficits in associative learning, which is also observed in humans. This deficit measured by impairment of eye blink classical conditioning was specific only to AD and were relieved upon application of substances that improve cognitive functions. Rabbit is only one existing AD model, in which cognitive impairment can be detected as the classically conditioned eye blink response (Woodruff-Pak, 2008).

Rabbits are useful animal models that allow to extrapolate animal studies to human. They contribute to the mechanistic studies of human disorders but are also involved in testing of new therapeutic compounds and development a new therapeutic techniques or strategies used in medicine.

2.5 Patterning of the human nervous system

The neural differentiation during embryogenesis leads to regional specification of the central nervous system (CNS) and formation of diverse neural subtypes. The initial patterning is controlled by signaling factors that form gradients along the dorso-ventral and antero-posterior axis. Additionally, migration of neuronal precursor cells is required for neural differentiation and pattern of synaptic connections. Neural subtype specification is controlled by many actions: cell-cell signaling, transcriptional regulation, gene expression, adhesion and motility activity. To make the story more complex: the same molecules can induce different effects depending on its concentration, receptor availability and modulation factors. In 1924 Spemann and Mangold showed that the formation of the nervous system in vertebrates is induced by signals that originate from a region of embryo called “organizer” (Spemann and Mangold, 1924). Later experiments indicated several molecules that induce neural tissue generation including noggin (Smith and Harland, 1992), chordin (Sasai *et al.*, 1995) and follistatin (Hemmati-Brivanlou *et al.*, 1994).

BMP signaling cascade similar as TGFB pathway activate the set of transcription factors (mainly SMADs) that consequently activate or repress the expression of the target genes (Schmierer and Hill, 2007). While inhibition of BMP pathway is required for effective neural differentiation, FGF2 activates ERK cascade (Kang *et al.*, 2005), which is relevant in neuroectoderm formation

(Rosa and Brivanlou, 2011). Combination of FGF2 and ActivinA regulate embryonic stem cell differentiation towards neural cells (Mimura *et al.*, 2015). The level of ACTIVIN/NODAL and BMP signaling influence on forebrain patterning (Lupo *et al.*, 2013). BMP inhibition is responsible for forebrain and midbrain specification, while FGF leads to posteriorization towards hindbrain/spinal cord identities (Lupo *et al.*, 2013). Retinoic acid (RA) plays crucial role in the first step of nervous system formation called primary neurogenesis. This molecule regulates proneural genes involved in primary neurons differentiation within the neuroepithelium (Bertrand *et al.*, 2002). RA is one of the first identified inductive signals in the paraxial mesoderm of the embryo. It promotes the differentiation of neural progenitor cells (NPCs) and stimulate expression of genes responsible for dorso – ventral spinal cord patterning (Del Corral *et al.*, 2003). RA represses ventral neuronal genes such as NK6 homeobox 1 (*Nkx6-1*) and sonic hedgehog (*Shh*) and induce dorsal genes: bone morphogenetic protein 4 (*Bmp4*), bone morphogenetic protein 7 (*Bmp7*), paired box 3 (*Pax3*) and *Wnt1* (Wilson *et al.*, 2004). Furthermore RA is involved in posteriorization of neuroectoderm (Kudoh *et al.*, 2002) and anterior – posterior patterning of the hindbrain (Glover *et al.*, 2006). RA acts in concentration-dependent manner, low concentration induces expression of anterior rhombomere markers: orthodenticle homeobox 2 (OTX2), empty spiracles homeobox 1 (EMX1), empty spiracles homeobox 2 (EMX2), distal-less homeobox 1 (DLX1), whereas higher concentration stimulate more posterior markers of the hindbrain: early growth response 2 (EGR2), WNT1, paired box 2 (PAX2) and homeobox (HOX) (Godsave *et al.*, 1998).

In the development of neural circuits WNT/ β -catenin pathway plays very important role. It interacts with transmembrane receptors and promotes self-renewal of neural progenitor cells (Machon *et al.*, 2007), while also responsible for development of cortex and hippocampus (Li and Pleasure, 2005).

Based on the above information the fate of single neuron depends on many factors including: position along the neuraxis, genetic profile and patterning factors. Unspecified progenitor cells within the neuroectoderm can differentiate into various neural subtypes by modulating signaling pathways in which are involved BMP, WNT, FGF, RA and other signaling molecules (**Figure 2**).

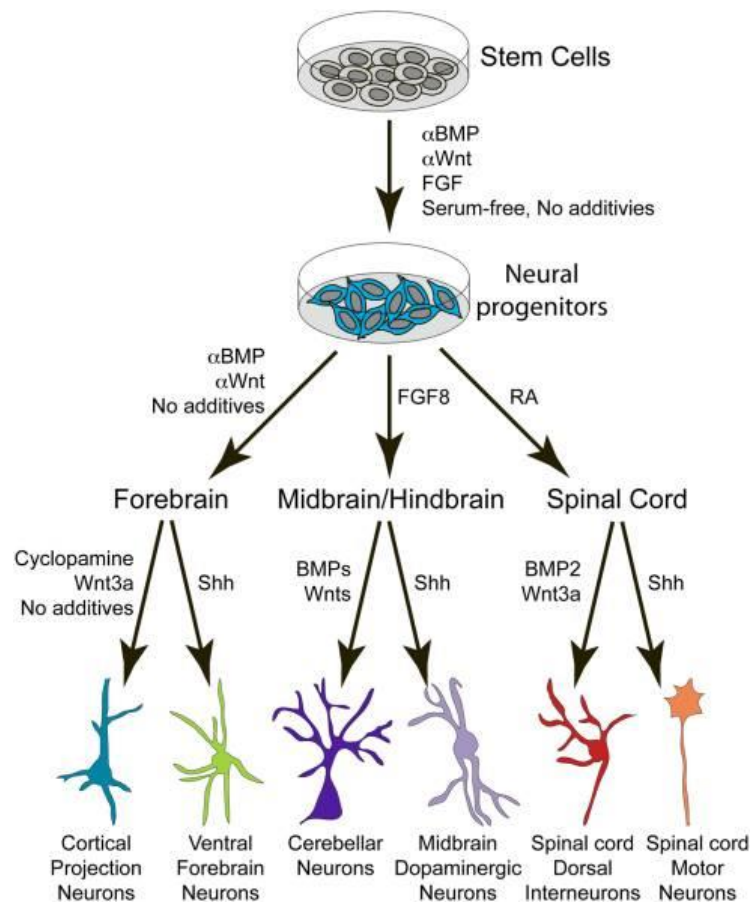


Figure 2. The scheme of the general procedures for deriving different neuronal subtypes from PSCs by using various secreted patterning factors: BMP4, WNT, FGF8 and RA.

(Adapted from Petros, et al., 2011)

2.6 PSCs in neurogenesis and neural differentiation *in vitro*

2.6.1 Generation of specific neural subtypes from PSCs *in vivo*

One of the crucial steps during neurogenesis is neuron formation. Neuronal progenitor cells located in the neuroectodermal layer induced by various signaling factors differentiate into neurons and glia cells. Based on the progenitor location in the developing brain, different neuronal and glia cells are produced. Progenitor cells located in the ventral neural tube generate motor neurons and oligodendrocytes while interneurons and astrocytes are produced from dorsal progenitor cells (Bertrand *et al.*, 2002). During nervous system formation *in vivo*, in the initial step of neural induction, the neural plate (sheet of ectodermal cells – neuroepithelium) is created from ectoderm cells (Levine and Brivanlou, 2007) (**Figure 3**). In the next stage neuroepithelium invaginates and fuses dorsally forming the neural tube, that contains multipotent neural stem cells (NSCs) throughout its rostro-caudal axis (Götz and Huttner, 2005). From NSCs various neurons and glia of the CNS are generated. Cell population migrating peripherally from the

dorsal neural tube form the neural crest, that give rise to the peripheral nervous system (PNS) (Nat *et al.*, 2007). In the rostral part of the neural tube 3 compartments of CNS develop: forebrain, midbrain and hindbrain, while the caudal neural tube forms the spinal cord. The human brain has enormously complex cellular diversity and connectivities fundamental to the neural functions. Its functional integrity relies on the precise production of diverse neuron populations and their assembly during development.

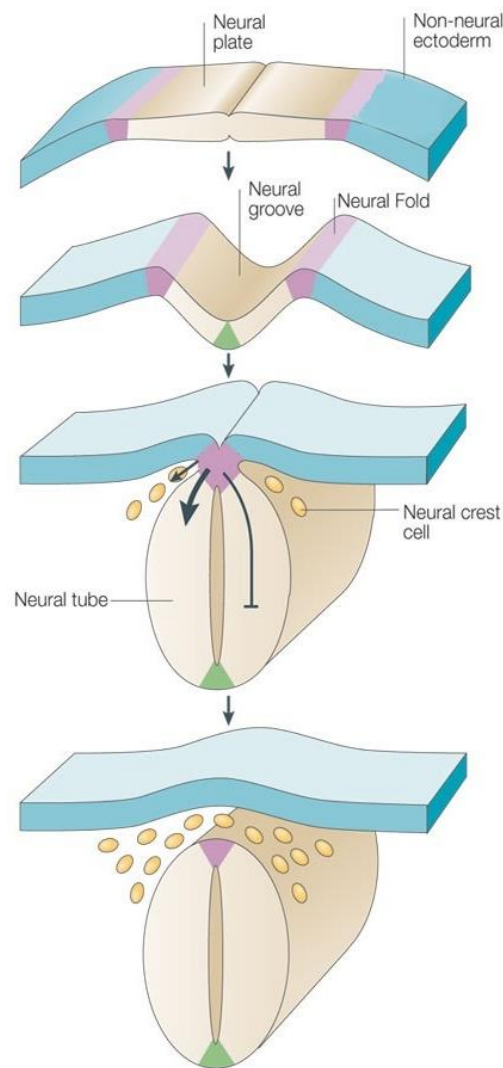


Figure 3. Formation of the nervous system. The CNS arises from a specialized epithelium, the neural plate. Folding of the neural plate to produce the neural groove is triggered by the formation of a distinct hinge point in the ventral region. At the end of neurulation, the lateral edges of the neural plate fuse and segregate from the non-neural epithelium to form a neural tube. Neural crest cells derive from the dorsal neural tube and migrate out to form the PNS, as well as melanocytes and cartilage in the head. (Modified from Liu and Niswander, 2005).

Neocortical neurons can be classified into subtypes according to their connectivity, morphology, physiology and molecular properties. In this way two major classes can be distinguished based on neurotransmitter expressions: glutamatergic projection neurons (approximately 80%) and GABA-ergic interneurons (approximately 20%), which create the sensory representation of the physical world. While interneurons connect in the vicinity to provide inhibition to the local circuit, projection neurons are excitatory and send axons to distant cortical and subcortical targets (Bartolini *et al.*, 2013). Although these two populations intermix within the mature neocortex, they are generated from different sectors of the telencephalon. Interneurons are produced from the ventral eminences (Anderson *et al.*, 2001), whereas projection neurons arise locally from the progenitors of the dorsal telencephalon (Gorski *et al.*, 2002).

Glutamatergic neurons and GABA interneurons, which belong to the neocortical neurons are generated from two different regions of the telencephalon. Glutamatergic neurons originate from dorsal telencephalic area (Gorski *et al.*, 2002) and form six layers of the cortex, whereas cortical GABA interneurons are born in the medial ganglionic eminence (MGE) and migrate to cortex and hippocampus (Anderson *et al.*, 2001). Development of glutamatergic neurons was shown to be associated with the basic helix-loop-helix (bHLH) transcription factor – neurogenin 2 (NEUROG2) (Hevner *et al.*, 2006). NEUROG2 contributes to promote the generation of glutamatergic neurons in the cortex and the hippocampus via a cascade of transcription factors involving paired box 6 (PAX6), T-box, brain 1 (TBR1) and T-box, brain 2 (TBR2) (Englund, 2005). It was indicated that NEUROG2 activates a cortical glutamatergic transcriptional pathway in the developing cortex and represses GABAergic transcription factors such as distal-less homeobox 2 (DLX2) (Fode *et al.*, 2000). Generation of GABAergic neurotransmitter phenotype in telencephalic neurons is regulated by Achaete-Scute family bHLH transcription factor 1 (ASCL1). This transcription factor activates DLX1/2 expression and specify a GABAergic phenotype through direct activation of glutamate acid decarboxylase (GAD) genes which encode the enzymes required for the synthesis of GABA neurotransmitter (Fode *et al.*, 2000).

Cholinergic neurons in the basal forebrain originate mainly from the MGE (Olsson *et al.*, 1998). During development, MGE express some transcription factors including LIM homeobox 8 (LHX8) (Matsumoto *et al.*, 1996) and gastrulation brain homeobox 1 (GBX1) that promote basal forebrain cholinergic neuron (bFCN) differentiation (Asbreuk *et al.*, 2002) and are upregulated by growth differentiation factor 2 (GDF2). It was also reported that cholinergic neuron development is stimulated by the presence of oligodendrocyte lineage transcription factor 2 (OLIG2), NK2 homeobox 2 (NKX2-2) and ASCL1 (Marin *et al.*, 2000; Furusho *et al.*, 2006). The survival of bFCNs is supported by neuronal growth factor (NGF) secreted from the cortex

and hippocampus, and brain-derived neurotrophic factor (BDNF), member of the neurotrophin family (NT). Survival of bFCNs is related with binding of NGF to high-affinity tyrosine kinase receptor TrkA and low-affinity neurotrophin receptor p75 (p75NTR) expressed by these neurons. Triggering of TrkA receptor by NGF leads to activation of PI3K/AKT, MEK/ERK and phospholipase C gamma 1 (PLCG1) signaling pathways. Active PI3K/AKT pathway inhibits apoptosis while MEK/ERK and PLCG1 stimulate proliferation by activation of MAPK pathway (Niewiadomska *et al.*, 2011).

The midbrain dopaminergic (mDA) neurons are born around day 10.5 of embryonic development (E10.5) and originate from the ventral midline of the neural tube floor plate (FP). Development of mDA is determined through signaling from the isthmic organizer (fibroblast growth factor 8, FGF8) and the notochord floor plate (SHH) (Ye *et al.*, 1998). Furthermore, the FGF8 expression is dependent upon WNT1, which activates engrailed homeobox 1 (*EN1*) and engrailed homeobox 2 (*EN2*) genes and is involved in differentiation of mDA progenitors towards specific mDA subtypes (Danielian and McMahon, 1996). Conversion of mitotic cells into postmitotic mDA precursors is induced by the mesodiencephalon ventricular zone (VZ) (Ono *et al.*, 2007). Developmental factors expressed in the VZ such as orthodenticle homeobox 1 (*OTX1*), *OTX2*, SHH and LIM homeobox transcription factor (*LMX*) define the cell fate along a dorsal and ventral axis (Smits *et al.*, 2006). Important role in the development, survival and maturation of the DA neurons play transcription factors: nuclear receptor subfamily 4 group A member 2 (*NR4A2*), LIM homeobox transcription factor 1 beta (*LMX1B*), paired like homeodomain 3 (*PITX3*), *EN1*, *EN2* and *NEUROG2* (Chinta and Andersen, 2005).

Motor neurons (MNs) are generated from progenitor cells in the ventral portion of the spinal cord. The identity of multiple MNs located throughout the longitudinal axis of the spinal cord is controlled by the HOX family of transcription factors (Dasen and Jessell, 2009). All MNs express canonical MN identity transcription factors such as ISL LIM homeobox 1 (*ISL1*) and motor neuron and pancreas homeobox 1 (*MNX1*) known as HB9. Additionally, they also express markers of a more mature and cholinergic MN phenotype including choline acetyltransferase (*ChAT*) and vesicular acetylcholine transporter (*VACHT*) (Son *et al.*, 2011). Generation of spinal MNs from PSCs require a three-stage process involving neuralization, caudalization and ventralization. SHH as a ventralizing factor promotes the expression of class II transcription factor including *OLIG2*, *NKX2-2* and *NKX6-1* (Patani *et al.*, 2011). RA responsible for caudalization stimulates the expression of class I transcription factors such as *PAX6*, paired box 7 (*PAX7*) and iroquois homeobox 3 (*IRX3*). MN precursors directly derived from the motor neuron progenitor (pMN) domain express *OLIG2*, *NKX6-1* and *NEUROG2* (Sander *et al.*,

2000). These transcription factors direct MN precursors to a post mitotic state and upregulate the level of MN genes such as HB9 (Nakano *et al.*, 2005).

2.6.2 Generation of specific neural subtypes from PSCs *in vitro*

Human ESCs and iPSCs can differentiate into functionally specialized cell types depending on the culture conditions and strictly defined factors composition. So far many different cell types were derived from hiPSCs. *In vitro* generation of neurons from hiPSCs show similar mechanism to *in vivo* development.

The first method for the derivation of bFCNs was based on the stimulation of hESCs with diffusible ligands present in the MGE at developmentally relevant time periods and then pretreatment of ESC-derived NPCs with SHH and FGF8 (Bissonnette *et al.*, 2011). Additional transient treatment with GDF2 caused a significant increase in the expression of bFCN markers such as: ChAT, acetylcholinesterase (AChE), nerve growth factor receptor TrkA and neurotrophin p75. Overexpression of LHX8 and GBX1 in NPCs pretreated with SHH/FGF8 resulted in the increased number of electrophysiologically functional bFCNs, co-expressing ChAT and p75 (Bissonnette *et al.*, 2011). Another study revealed that stimulation of NPCs with neurotrophins such as BDNF, NGF, ciliary neurotrophic factor (CNTF) and neurotrophin3 (NT3) resulted in significantly higher growth of ChAT positive neurons (Nilbratt *et al.*, 2010). The latest data has reported an efficient production of cholinergic neurons using a culture of embryoid bodies in three-dimensional system without any additional factors. Newly generated bFCNs expressed Tubulin Beta 3 Class III (TUBB3), CHAT, ISL1 and p75 and were able to integrate into the adult rat brain after transplantation (Crompton *et al.*, 2013).

Dopaminergic neurons (DA) were first successfully generated from hESCs using FGF8 and SHH (Ye *et al.*, 1998). Additional presence of glial cell derived neurotrophic factor (GDNF), known as dopaminergic neuroprotectant factor in the culture medium, increased DA neuron differentiation (Young *et al.*, 2010)(Young *et al.*, 2010).

Rapid and efficient conversion of hESCs and iPSCs into neurons by the synergistic action of two inhibitors of SMAD pathway: NOGGIN and SB431542 has been reported by Chambers *et al.* in 2009. Inhibition of SMAD pathway by SB431542 is associated with decrease of NANOG level and increase in the expression of caudal type homeobox 2 (CDX2) that consequently leads to loss of pluripotency and differentiation through the trophoblast lineage. Contrary to SB431542, NOGGIN downregulates CDX2 expression and represses BMP release, that drives trophoblast destiny. Efficient neural induction of hESCs and iPSCs under adherent culture conditions is possible only by dual inhibition of SMAD pathway (Chambers *et al.*, 2009b).

Culture of the induced NPCs in medium supplemented with BDNF and GDNF results in generation of mixed neuronal cell population composed mainly of GABA and glutamatergic neurons (Manuilova *et al.*, 2001). Directed differentiation of NPCs towards specific neuronal lineage may be accomplished with an appropriate growth and differentiation factors. For instance, exposure of NPCs to SHH and FGF8, and then culture in media supplemented with BDNF, GDNF, ascorbic acid (AA), transforming growth factor beta 3 (TGFB3) and cyclic adenosine 3',5'-monophosphate (cAMP) results in production of tyrosine hydroxylase (TH) positive neurons co-expressing TUBB3 (Chambers *et al.*, 2009b).

In the last decades many multiple approaches have been designed to direct differentiation of mouse and human PSCs into MNs. Most of the current protocols for derivation of motor neurons are based on embryoid body mediated neural induction followed by exposure to defined morphogens such as SHH and RA (Li *et al.*, 2005; Singh *et al.*, 2005). Time course studies revealed that mESCs and hESCs exposed to SHH and RA generate motor neurons. Moreover the first HB9⁺ MNs begin to appear from 3 to 5 days after addition of patterning factors (Wichterle *et al.*, 2002). Compared to mESC, generation of MNs from hESCs is more protracted, and require 2-4 weeks of additional maturation to get an electrophysiologically active, HB9⁺ ISL1⁺ neurons (Lee *et al.*, 2007; Chambers *et al.*, 2009b; Amoroso *et al.*, 2013). Studies on mouse and human PSCs indicated that endogenous WNT and FGF signaling as well as inhibition of BMP/ACTIVIN/NODAL signaling cascade promote differentiation of MNs with caudal positional identity (Peljto *et al.*, 2010; Patani *et al.*, 2011). Physiological recordings have demonstrated that PSC-derived MNs exhibit many electrophysiological properties relevant to motor neuron functions. *In vitro* generated MNs respond to GABA, glutamate and glycine and increase inward currents indicating the expression of proper receptors and the correct response to the stimuli (Hester *et al.*, 2011; Son *et al.*, 2011; Amoroso *et al.*, 2013).

Successful generation of cortical neurons was determined mostly by coordination of SHH and WNT signaling (Li *et al.*, 2009; Xu *et al.*, 2010; Liu *et al.*, 2013). It was reported that hESCs differentiated towards dorsal and ventral telencephalic progenitors and then formed functional glutamatergic and GABAergic neurons, respectively. In the presence of endogenous WNT signaling ESCs differentiated to primitive dorsal telencephalic precursors, which was achieved through upregulation a truncated form of GLI family zinc finger 3 (GLI3), a SHH repressor. The inhibition of WNT protein by Dickkopf WNT signaling pathway inhibitor 1 (DKK1) or activation of SHH signaling led to completely conversion of dorsal precursors to ventral progenitors. This was attributed to downregulation of the truncated GLI3 and upregulation of active, full-length GLI3 expression (Li *et al.*, 2009). Another study revealed that combining

retinoid signaling and inhibition of SMAD pathway by Dorsomorphin/NOGGIN and SB431542 promote generation of cerebral cortex progenitor cells with almost 90% efficiency. Derived in this method neural stem cells demonstrated expression of forkhead box G1 (FOXP1), EMX1 and TBR2 (Shi *et al.*, 2012).

2.7 PSCs in modelling of neurological diseases

In the majority the underlying mechanisms of neurological dysfunction are not yet fully examined. Most of the current knowledge about neurodevelopmental and neurodegenerative disorders is based on the studies in postmortem nerve tissues and brain. Due to the limited potential of neuronal samples from postmortem organs and the inability to examine live neurons understanding the cellular and molecular mechanism of such diseases is very restricted. In addition, studies of tissues from autopsy that often represent the end stage of the disease do not always correspond with the course of disease. A significant contribution to elucidating the pathogenesis of various neurological abnormalities have transgenic animal models that can mimic human diseases (Young, 2009). Well-designed transgenic/knockout technology provides a useful tool for investigating of disease mechanism. However, animal models do not fully recapitulate complex human disease phenotype and are limited mainly to monogenetic disorders. Recent advances in pluripotent stem cell technology give a new opportunity to overcome these limitations. Stem cell derived specific neurons can be a perfect tool in human disease modelling, drug screening and treatments for neurological abnormalities. Here, we collected the existing human iPSC-based models which are used for the study of different neurological disorders (**Table 1**). This data well presents the process that the number of models is increasing exponentially and the most relevant and devastating diseases are in the forefront of the interests.

2.7.1 Modelling of neurodevelopmental disorders

Neurodevelopmental diseases are associated with some degree of neuropathology, usually involving differences in the elaboration of neuronal morphology, branching and connectivity. So far many research groups have reported modelling of these disorders such as Down's syndrome (DS), Rett syndrome (RTT) or amyotrophic lateral sclerosis (ALS) using iPSCs.

Down's syndrome (DS), also known as Trisomy 21 is the most common genetic disorder related with the dosage imbalance of the genes located on HSA21 leading to neuropathological defects such as alterations in neurogenesis and synaptogenesis (Nadel *et al.* 2003). Neurodevelopment impairment in DS was modeled with iPSCs derived from second trimester amniotic fluid cells (TS21 AF-iPSCs). NPCs produced from TS21-iPSCs contained three pairs of chromosomes 21

(TS21). In comparison to normal AF-iPSCs, mutant cells have shown increased level of APP and poorly developed neural network.

Table 1. Neurological disorders modeled with patient specific human induced pluripotent stem cells (Adapted from Ochalek *et al.*, 2016).

Disease	Genetic background	Disease related phenotype	Affected neurons	iPSCs model	References
Neurodegenerative disorders					
Alzheimer's disease	PS1, PS2, APP duplication, APOE	- increased A β 42 secretion; - A β plaque formation; - increased phospho-Tau; - increased GSK3 β activity	basal forebrain cholinergic neurons; cortical neurons	- AD iPSCs with E693 deletion in APP gene; - AD iPSCs with mutation in APP (V717I); - AD iPSCs with a duplication APP gene; - AD iPSCs with PS1 (A246E) and PS2 (N141I) mutation	(Yagi <i>et al.</i> , 2011; Israel <i>et al.</i> , 2012; Kondo <i>et al.</i> , 2013; Muratore <i>et al.</i> , 2014)
Parkinson's disease	SNCA, LRRK2, PARKN, PINK1, UCHL1, GBA	- α -synuclein accumulation; - reduced numbers of neuritis; - increased susceptibility to oxidative stress; - accumulation of ER-associated degradation substrates	dopaminergic neurons	- PD iPSCs with triplication of the SNCA; - PD iPSCs with α -synuclein mutation (A53T); - PD iPSCs with G2019S mutation in LRRK2 gene; - PD iPSCs with mutation in PINK1	(Bohnen and Albin, 2011; Nguyen <i>et al.</i> , 2011; Seibler <i>et al.</i> , 2011; Chung <i>et al.</i> , 2013)
SMA	SMN1, SMN2	- reduced SMN gene expression; - Fas ligand-mediated apoptosis of MN; - increased level of caspase-3, caspase-8 and membrane-bound Fas ligand; - reduced size, axonal elongation and neuromuscular junction production	motor neurons	- iPSCs with SMN1 mutation from SMA type I patients	(Ebert <i>et al.</i> , 2009; Faravelli <i>et al.</i> , 2014)
ALS	SOD1, TDP-43, FUS, VAPB	- neurofilament-L aggregation in neuritis; - axonal degeneration; - increased secretion of TDP-43; - exhibited shortened neurites	motor neurons	- ALS iPSCs with A4V SOD1 mutation; - ALS iPSCs with D90A SOD1 mutation; - ALS iPSCs with mutation invTDP-43 gene; - ALS iPSCs with VAPB (P56S) mutation	(Bilican <i>et al.</i> , 2012; Aliaga <i>et al.</i> , 2013; H. Chen <i>et al.</i> , 2014)
Huntington's disease	HTT (CAG repeats)	- increased vulnerability to cell stressors and BDNF withdrawal; - impaired lysosomal activity; - mitochondrial fragmentation; - alterations in transcription repressor activity; - enhanced caspase 3/7 activity	cortical neurons; GABAergic medium spiny neurons	- iPSCs with HTT mutation from homozygous and heterozygous HD patients HD72-iPSC in the YAC128 model of HD	(Le Goff <i>et al.</i> , 2006; Park <i>et al.</i> , 2008; An <i>et al.</i> , 2012; Jeon <i>et al.</i> , 2014; Ross and Akimov, 2014)

Neurodevelopmental disorders

Familial Dysautonomia	IKBKAP	- reduced IKAP protein level; - cell migration deficiency; - defects in neurogenic differentiation; - decreased in number of myelinated small fibers and intermediolateral column neurons	sensory neurons; autonomic neurons	- FD iPSCs with mutation in IKBKAP gene	(Lee <i>et al.</i> , 2009)
Rett syndrome	MECP2e1, MECP2e2	- reduced soma size; - altered dendritic spine density; - dysfunction in action potential; - alterations in synaptic function; - defects in synaptic plasticity	glutamatergic neurons	- RS iPSCs with MECP2 mutation	(Farra <i>et al.</i> , 2012; Djuric <i>et al.</i> , 2015)
ASD	NLGN1, NLGN3, SHANK2, SHANK3, NRXN1, NRXN3	- reduced glial differentiation; - altered gene expression related to cell adhesion and neuron differentiation; - deficits in neuronal specification, synapse formation and excitatory neurotransmission	cortical neurons	- ASD iPSCs with functional knockdown of NRXN1 gene; - ASD iPSCs with SHANK3 deletion	(Zeng <i>et al.</i> , 2013; Kim <i>et al.</i> , 2014)
Down syndrome	trisomy of chromosome 21 (HSA21)	- alterations in neurogenesis and synaptogenesis; - elevated APP and A β 42 expression; - Tau protein hyperphosphorylation; - poorly developed neural network; - overproduction of reactive oxygen species	neurons in the brain	- DS iPSCs with three pairs of chromosomes 21 (T21-iPSCs); - isogenic iPSCs from DS individuals; - DS iPSCs with trisomy 21 deletion through TKNEO; - DS iPSCs with trisomy 21 deletion through Xist	(Park <i>et al.</i> , 2008; Li <i>et al.</i> , 2012; Jiang <i>et al.</i> , 2013; Chen <i>et al.</i> , 2014)
Schizophrenia	DISC1	- decreased neuronal connectivity; - synaptic deficits; - PSD95 downregulation; - fewer neurites	neurons	- iPSCs from schizophrenia patients; - SZ iPSCs with a mutation in DISC1 gene	(Thomson <i>et al.</i> , 2013; Brennand <i>et al.</i> , 2014)

DS neurons overexpressed miR-155 and miR-802 that caused the inhibition of the methyl-CpG binding protein 2 (MECP2) target gene expression (Lu *et al.*, 2012). Weick *et al.* showed that iPSCs with full trisomy of chromosome 21 and control iPSCs differentiate with similar efficiency generating functional dorsal forebrain neurons. Genomic profiling of TS21 iPSC derived neurons revealed changes in HSA21 genes consistent with the presence of 50% more genetic materials as well changes in non-HSA21 genes in human chromosome 21 compared with control (Weick *et al.*, 2013). Additionally, TS21 neurons exhibited lower synaptic activity which affect excitatory and inhibitory synapses equally. Furthermore, DS neurons indicated higher sensitivity to oxidative stress induced apoptosis that can contribute with accelerated neurodegeneration observed in DS brain (Briggs *et al.*, 2013).

Rett Syndrome (RTT) is a postnatal progressive neurodevelopmental disorder that is linked with the impairment of the number of axonal boutons and axonal arborization suggesting a decrease in the overall number of synapses in RTT brains (Belichenko *et al.*, 2009). More than 99% of RTT cases are sporadic, and all remaining are caused by mutations in MECP2 gene. Analysis of neurons generated from the first patient iPSC model of RTT has demonstrated that they have a

molecular signature of cortical neurons. MECP2 mutant neurons showed decreased soma size, altered dendritic spine density and a dysfunction in action potential generation: voltage-gated Na⁺ currents and miniature excitatory synaptic current frequency and amplitude (Djuric *et al.*, 2015). The cellular defects of RTT neurons can be reversed in glutamatergic synapses by insulin like growth factor 1 (IGF1) and gentamicin. Both drugs are considered to be a candidate for pharmacological RTT treatment. Moreover, RTT-iPSCs carrying MECP2 mutations increase the frequency of neuronal L1 (long interspersed nuclear elements-1) retrotransposition. This unexpected fact suggests that also pre-symptomatic defects can be a good biomarker for early disease detection. Farra *et al.* have described neuronal differentiation of iPSCs derived from MECP2-deficient mice. MECP2-deficient neurons showed some disruptions in evoked action potentials generation and glutamatergic synaptic transmission. Compare to the wild type neurons they fired fewer action potentials and displayed decreased action potential amplitude (Farra *et al.*, 2012). Studies on human and mouse iPSC suggest that defect in Na⁺ channel function may contribute to the disturbances in RTT neuronal network (Marchetto *et al.*, 2010).

Amyotrophic lateral sclerosis (ALS) affects the motor neurons and the corticospinal neurons of the motor cortex and only 5% of all cases are familiar and related with mutations in several genes such as superoxide dismutase 1 (SOD1), TAR DNA binding protein (TARDBP), FUS RNA binding protein (FUS) and VAMP associated protein B and C (VAPB). Recently researches created ALS-disease models including iPSC derived MNs from patients with A4V SOD1 and D90A SOD1 mutations. Generated ALS neurons contained small SOD1 aggregates in cytoplasm and nuclei, and exhibited neurofilament-L (NF-L) aggregation in cytoplasm and neurites leading to axonal degeneration (Chen *et al.*, 2014). Another study demonstrated that MNs derived from ALS-iPSC patients with TARDBP mutation formed cytosolic aggregates, increased secretion of soluble and detergent resistant TARDBP protein and exhibited shortened neurites and increased vulnerability to cell stressors (Bilican *et al.*, 2012; Egawa *et al.*, 2012). Furthermore, it was found that TARDBP mutations leads to abnormal axonal transport (Alami *et al.*, 2014). iPSC models were also generated from ALS patients with mutation in VAPB gene. MNs derived from ALS8-iPSCs demonstrated reduced level of VAPB protein without the presence of cytoplasmic aggregates (Mitne-Neto *et al.*, 2011). In 2013, Burkhardt *et al.* successfully produced MNs from sporadic ALS. These neurons showed *de novo* TARDBP aggregation in lower motor neurons and upper motor neuron-like cells recapitulating pathology in postmortem tissues from which they were generated (Burkhardt *et al.*, 2013).

2.7.2 Modelling of neurodegenerative disorders

Neurodegenerative diseases are often defined as hereditary and sporadic conditions that are characterized by progressive nervous system dysfunction. These disorders are usually associated with atrophy of the affected central or peripheral structures of the nervous system.

Parkinson's disease (PD) is one of the most common progressive neurodegenerative disorder associated with degeneration of dopamine containing neurons in the *substantia nigra pars compacta* and a loss of dopamine in the striatum leading to motor impairments (Dawson, 2000). The majority of PD cases are classified as sporadic and only about 10-20% of patients report a familial monogenic form of disease, related mostly with mutations in a group of genes: synuclein alpha (SNCA), PTEN induced putative kinase 1 (PINK1), Parkin RBR E3 ubiquitin protein ligase (PARK2), leucine rich repeat kinase 2 (LRRK2), glucosylceramidase beta (GBA), and ubiquitin C-terminal hydrolase L1 (UCHL1) (Lill *et al.*, 2016). One of the first model of familial PD based on iPSC-derived neurons from a patient with triplication of the SNCA gene has been reported by Byers *et al.* Generated by this group DA neurons expressed higher level of SNCA and were more susceptible to oxidative stress (Byers *et al.*, 2011). Another neurons derived from patients with A53T mutation in SNCA exhibited higher production of nitric oxide and 3-nitrotyrosine (3-NT), and accumulated more ER-associated degradation substrates (Chung *et al.*, 2013). Neurons carrying the mutation in PARK2 upregulated monoamine oxidase expression and block dopamine uptake that results in higher dopamine secretion (Jiang *et al.*, 2012). Other researchers have reported that iPSCs derived from either sporadic PD or LRRK2 PD differentiate into DA neurons with similar phenotypes including overexpression of SNCA and inhibition of neurite outgrowth (Sánchez-Danés *et al.*, 2012). If the above results will be reproducible, the iPSCs can be also use in modelling of sporadic (idiopathic) PD cases. Due to unknown causes of the disease, it is still not well examined whether neurons derived from idiopathic PD patients reveal the same phenotype related to PD *in vitro* (Soldner *et al.*, 2009). Only a small percentage of sporadic PD is associated with known genetic factors whereas the mechanism leading to majority of them can be an effect of combination of genetic and environmental factors. Thus, an increased number of analyzed cell lines is required to generate more reliable iPSC models especially in the case of complex disorders such as sporadic PD.

Huntington's disease (HD) is an incurable, hereditary brain disorder caused mainly by a heterozygous expanded trinucleotide repeat (CAG)_n, encoding glutamine in huntingtin (HTT) gene. At the pathological level, HD affects medium spiny GABA neurons located in the part of the *corpus striatum* region called *caudate-putamen* (Waldvogel *et al.*, 2014). Additionally, HD neurons contain intranuclear inclusion bodies as well as perinuclear and neuritic aggregates of

HTT protein. Some studies revealed that also cholinergic system is impaired in HD patients. Decreased activity of ChAT and reduced ACh secretion were observed in the striatal tissue of the PD brain (Smith *et al.*, 2006). *In vitro* studies indicated that iPSCs derived from HD patients and differentiated into mature neurons display many phenotypic features of the disease such as impaired lysosomal functions, alterations in transcription repressor activity and mitochondrial fragmentation (Ross and Akimov, 2014). Additionally, HD neurons exhibited changes in metabolism and gene expression (e.g. cadherin pathway and TGFB pathway), abnormalities in oxygen consumption and higher susceptibility to cellular stress inducers and BDNF withdrawal (An *et al.*, 2012). Data published by Park *et al.* revealed higher activity of caspase 3/7 in striatal neurons derived from HD iPSCs (Park *et al.*, 2008). However, treatment of HD iPSCs with inhibitor of mitochondrial fission related protein: dynamin 1like (DNM1L) reduced neuronal cell death (Bilican *et al.*, 2012). Jeon *et al.* revealed that HD-derived neuronal progenitor cells upon injection into rats with excitotoxic striatal lesions recover behavioral health, even though the transplanted cells displayed HD phenotype (Jeon *et al.*, 2012), suggesting important role of mutation correction in iPSCs for effective treatment of HD.

2.8 PSCs in modelling of Alzheimer's disease

There are nearly 47 million people living with dementia worldwide, which is predicted to double every 20 years increasing to more than 131 million by 2050. Alzheimer's disease (AD) is the best characterized among them which accounts for 50-60% of all dementia cases (Prince *et al.*, 2016). This common neurodegenerative disease is clinically characterized by a progressive and gradual cognitive impairment, synapse loss and substantial loss of neurons in later stages. Due to the disease heterogeneity, the etiology of AD is still not very well understood. Most cases of early onset AD are linked to autosomal dominant inherited mutations in the genes encoding *APP*, *PSEN1* and *PSEN2*. These cases are referred to as familial AD (fAD) and are well characterized. In contrary, the etiology of the remaining 95% late onset AD, often referred to as sporadic AD (sAD), requires further investigation, due to the various factors involved in the pathology, including genetic and environmental exposures (Borenstein *et al.*, 2006). Moreover, cellular changes in the brain precede the first clinical symptoms by 10-15 years, and there is a lack of early diagnostic biomarkers for the prodromal stages of AD. Currently, there is no cure against AD and the available medications can only slow down the progression of dementia and slightly improve the life quality of the patients (Selkoe, 2013). Two well defined pathological hallmarks

of AD have been described: the formation of extracellular amyloid plaques and NFTs formed by aggregated hyperphosphorylated TAU protein (Murphy and LeVine, 2010).

2.8.1 TAU pathology

Human TAU, a microtubule associated protein TAU (MAPT) is broadly expressed in the central and PNS and present also in lung, testis and kidney. TAU was found in the cytosol, but is also associated with the cell membranes. Although TAU is mainly expressed in axons, it appears as well in somatodendritic compartments and oligodendrocytes (Mietelska-Porowska *et al.*, 2014). The human gene encoding *MAPT* consisting of 16 exons, is located on the long arm of chromosome 17 (17q21) (Andreadis *et al.*, 1992). TAU protein in human brain is consists of six isoforms as a result of alternative mRNA splicing (Goedert *et al.*, 1989). Biophysical studies revealed that hydrophilic TAU exists in natively unfolded or intrinsically disordered form and its polypeptide chains are highly flexible and display a low content of secondary structures (Jeganathan *et al.*, 2008). Primary structure analysis has shown the presence of three main domains in TAU molecule: an acidic N-terminal part; a proline-rich region and a basic C-terminal domain (**Figure 4**). N-terminal domain of TAU does not bind microtubules but projects away from the microtubule surface and is named projection domain. This part of the protein is involved in the interactions with other cytoskeletal elements, neuronal plasma membranes or mitochondria and determines spacings between microtubules in axons and dendrites (Hirokawa *et al.*, 1988). The middle part of TAU termed proline rich domain can bind proteins with the Src-homology 3 (SH3) domains and contains numerous proline residues that target various proline directed kinases (Mandelkow, 2012). The C-terminal part contains the microtubule binding repeats (MTBR) (R1 – R4) which bind to microtubule and promote their assembly. Furthermore, the recent studies confirm the role of MTBR in the modulation of TAU phosphorylation (Sontag *et al.*, 1999).

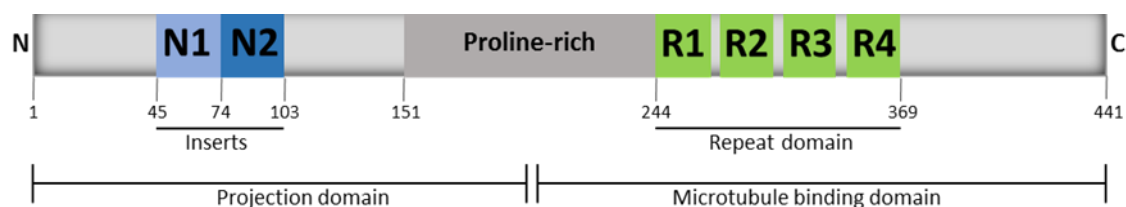


Figure 4. Schematic representation of human TAU isoform (441 amino acids) with the functional projection and microtubule binding domains. This isoform contains two N-terminal inserts of 29 amino acids (N1 and N2) in combination with four C-terminal repeat regions (R1, R2, R3 and R4).

Human TAU is a multifunctional protein that interacts with many binding partners such as signaling molecules, lipids and cytoskeletal elements (Jho *et al.*, 2010). The most important function of TAU is its role in tubulin polymerization, in which binding of TAU to tubulin is controlled by posttranslational modifications especially phosphorylation. The proper TAU-tubule interactions are crucial in axonal transport and binding of motor proteins: dyneins and kinesins (Dixit *et al.*, 2008). Moreover, TAU can bind to cytoskeletal components, spectrin and actin filaments, and lead to stabilization of microtubule – neurofilament connections that reduce the microtubule lattice flexibility (Farias *et al.*, 2002). TAU may also act as a scaffold protein in neurons by modulating the activity of SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase (SRC) and in oligodendrocytes, where connect microtubules with FYN proto-oncogene, Src family tyrosine kinase (FYN) to support process extension (Klein *et al.*, 2002). Additionally, TAU is involved in growth factor signaling (Niewiadomska *et al.*, 2005). For instance, this molecule can increase the activity of MAPK facilitating signaling through NGF and epidermal growth factor receptors (EGFR). TAU is highly regulated by post-translational modifications including phosphorylation, acetylation, glycation, nitration, polyamination, oxidation, ubiquitination and some other modifications as reviewed in (Mietelska-Porowska *et al.*, 2014). Phosphorylation is the most common post-translational TAU modifications and is the effect of dynamic regulation of TAU kinases and TAU phosphatases (**Figure 5**). A normal level of phosphorylation is required for proper cytoskeletal organization, axonal transport, cell signaling and neuronal outgrowth. Abnormal phosphorylation (hyperphosphorylation) triggers the pathological aggregation of TAU and leads to lose its biological activity which consequently results in tauopathy observed in many neurodegenerative disease such as frontotemporal dementia (FTD) and parkinsonism linked to chromosome 17 or AD.

Studies on normal human brain tissue revealed phosphorylation of TAU at several serine and threonine residues whereas in AD brain more than 40 phosphorylation sites have been identified (Gong *et al.*, 2005). The phosphorylation level of TAU is around 3-4 times higher in AD patients compare to healthy individuals. Most of the potential TAU phosphorylation sites are located at the MTBD in the proline rich regions (**Figure 4**). These regions include serine and threonine residues in SP and TP motifs and therefore they are targets of 3-phosphoinositide dependent protein kinases (PDPK) such as: mitogen-activated protein kinase 1 (MAPK1), mitogen-activated protein kinase 8 (MAPK8), cyclin-dependent protein kinase 1 (CDK1), cyclin-dependent protein kinase 5 (CDK5) and GSK3B (Gong and Iqbal, 2008a). Other non-proline sites containing the Lysine-Xaa-Glycine-Serine (KXGS) motifs are phosphorylated by microtubule affinity-regulating kinase (MARK) or protein kinase AMP-activated catalytic

subunit alpha 2 (PRKAA1) (Drewes *et al.*, 1997). Hyperphosphorylation of TAU by the above kinases may impair microtubule integrity in AD patients. Studies have clearly demonstrated that phosphorylation of TAU by GSK3B and CDK reduces the microtubule affinity of TAU (Wagner *et al.*, 1996) whereas phosphorylation of the serines within the KXGS motifs destructively affects TAU-microtubule interactions (Drewes *et al.*, 1997). Phosphorylation of TAU at different epitopes has different impact on its biological function and pathological role. A quantitative *in vitro* studies revealed that phosphorylation of TAU at Thr231, Ser235 and Ser262 reduces binding capacity on microtubules by 35%, 25% and 10% respectively (Sengupta *et al.*, 1998). Phosphorylation at epitopes such as: Ser199/Ser202/Thr205, Thr212, Thr231, Thr231/Ser235, Ser235, Ser262, Ser262/Ser356, Ser396 and Ser422 results in diminishment of TAU activity and microtubule destabilization (Gong and Iqbal, 2008b). Moreover, phosphorylation at Thr231, Ser396 and Ser422 promotes self-aggregation of TAU into filaments.

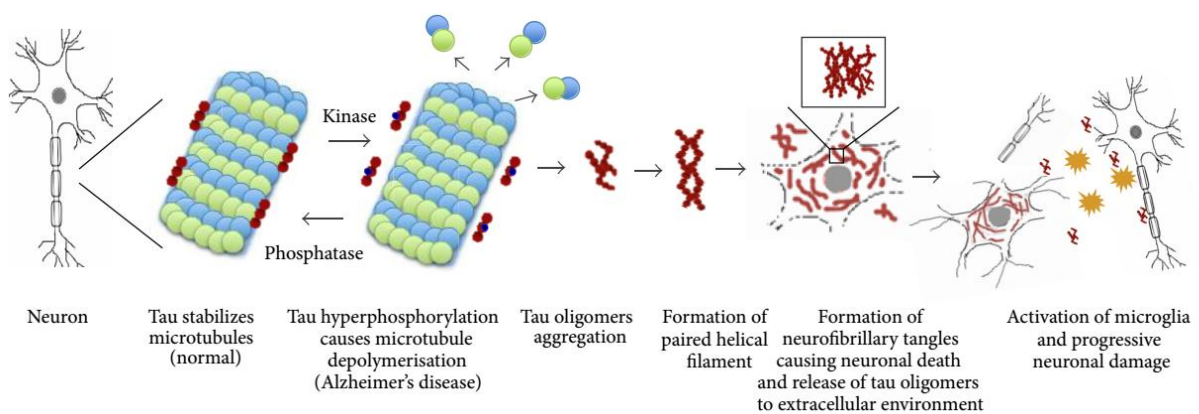


Figure 5. Role of TAU protein in stabilization of microtubules. Abnormal phosphorylation of TAU causes microtubule depolymerization and formation of insoluble TAU oligomers. (Adapted from Mokhtar *et al.*, 2013)

Abnormally phosphorylated TAU within specific neuronal populations form paired helical filaments (PHFs) that aggregate into intracellular fibrillar structures – NFTs. TAU filaments form fine neuropil threads or bundles of PHFs that accumulate in dystrophic neurites or neuronal bodies respectively, and are released as extracellular tangle after cell death. According to the recent studies, the number and localization of NFTs correlate with the disease progression (Arriagada *et al.*, 1992). Structural analysis of PHFs demonstrated high phosphorylation of TAU at residues localized at C-terminal domain that correlate with a loss of microtubule-binding capacity and TAU accumulation in neuronal bodies. Nowadays, several mechanisms have been proposed to explain neurotoxic effect of PHF-TAU. First, it was shown that phosphorylated

TAU causes disassembly of microtubules and leads to loss or decline of axonal and dendritic transport. Secondly, PHF-TAU disrupts intracellular compartments that are involved in normal metabolism. Thirdly, TAU aggregates change cellular shape, retards cell growth and impair a movement of microtubule-dependent motor proteins that disrupt transport of different organelles within the cell (Avila, 2006). The above observations indicate that phosphorylation and aggregation of TAU is one of the main pathogenic mechanisms directly associated with neurodegeneration.

2.8.2 Amyloid plaques

Another pathological hallmark of AD is accumulation of A β plaques, composed of extracellular insoluble fibrous amyloid aggregates derived from the aberrant cleavage of the transmembrane protein APP. The major structural element found in amyloid deposits is the β -sheet, therefore the structure of protein forming plaques is altered and displays inappropriate folding (Kirschner *et al.*, 1986). It was documented that A β accumulation is a critical AD event which starts a pathogenic cascade promoting synaptic loss, neuronal death and dementia (Hardy and Selkoe, 2002).

APP is an integral membrane protein with high affinity to copper, and in the physiological environment plays important role in neurodevelopment and neuronal growth (Luo *et al.*, 1996). It has been reported that APP participate in synaptogenesis, signal transduction, regulation of neurite outgrowth, neuronal cell-cell and cell-surface adhesion, and cell migration (Breen *et al.*, 1991; Small *et al.*, 1994). Furthermore, C-terminal part of APP is involved in gene expression and neuronal survival (Nguyen *et al.*, 2008). Direct product of APP cleavage, A β also plays many important physiological roles. The level of A β peptide in human brain is dynamically regulated by synaptic activity. Due to the ability to capture redox metal, A β can prevent binding of Cu, Fe or Zn with other ligand in redox cycle and act as a chelator and antioxidant (Atwood *et al.*, 2003). Recent studies revealed that A β exhibits dual effects on neuronal cells: neurotrophic or neurotoxic. These effects may contribute with a peptide concentration, age of the individuals or environmental conditions. Low concentration of A β is crucial in regulation of synaptic plasticity and improvement of cognitive functions, whereas highly concentrated A β together with an age-based effect may lead to dysregulation and loss of synaptic functions (Shankar *et al.*, 2007). Moreover, physiological level of soluble A β is involved in neuronal growth, cellular survival, learning and memory process (Garcia-Osta and Alberini, 2009).

There are two pathways for processing APP: an amyloidogenic pathway and a non-amyloidogenic, constitutive secretory pathway (**Figure 6**). In the non-amyloidogenic pathway

APP is cleaved by α -secretase within the A β domain in the process called ectodomain shedding. This APP cleavage results in production of a soluble extracellular fragments of sAPP α , that exhibit neurotrophic and neuroprotective functions (Edwards *et al.*, 2009) and membrane-bound APP C-terminal fragment α (CTF α). α -secretase activity is detected in disintegrin and metalloproteinases such as ADAM metallopeptidase domain 9, -10 and -17 (ADAM9, ADAM10, ADAM17). However recent study indicates that ADAM10 is the major α -secretase involved in the ectodomain shedding process in the brain (Kuhn *et al.*, 2010). In the next step γ -secretase complex including nicastrin, Aph-1 homolog A, gamma-secretase subunit (APH1A), presenilin enhancer 2, PSEN1 and / or PSEN2, cleaves CTF α to produce the non-amyloidogenic P3 fragment and the APP intracellular domain (AICD). In contrast, the proamyloidogenic pathway involves cleavage of APP by β -secretase 1 (BACE1), leading to generation of the N-terminus of A β and sAPP β ectodomain (**Figure 6**). The resulting membrane bound APP C-terminal fragment β (CTF β) is then cut by γ -secretase to produce heterogeneous C-terminal fragments of A β (A β 37, A β 38, A β 40, A β 42, A β 43) and AICD (De Strooper *et al.*, 2010). Secreted from neurons A β self-aggregates into extracellular plaques and leads to pathophysiological changes in AD. The most abundant isoform in normal and AD brain is A β 40 (~80-90% of the total A β peptide), whereas the A β 42 variants (~5-10%) are the most deleterious due to more hydrophobic and fibrillogenic properties (Selkoe, 2001). Another cleavage product, AICD has been reported to be involved in signaling from the membrane to the nucleus through epigenetic modulation of gene expression (Nalivaeva and Turner, 2013). On the other hand, AICD can be further processed by caspase generating a CT31 fragment, which is a potent apoptosis inducer (Lu *et al.*, 2000). BACE1 and ADAM10 compete with each other to cut APP, thus higher activity of BACE1 causes decreased α -secretase processing of APP and *vice versa*. Increased ADAM10 activity corresponds with reduction of BACE1 cleavage and increased generation of A β (Postina *et al.*, 2004). Higher A β level was also observed in PSEN1 and PSEN2 mutants which display higher activity of γ -secretase. The above observations may be implicated in the therapeutic strategies that increase activity of ADAM10 can reduce cleavage of APP by β -secretase and decrease A β release.

Although A β oligomers are relatively non-pathogenic, soluble A β peptide species are synaptotoxic and may prune dendritic spines, disrupting the memory-encoding network in the entorhinal cortex, the parahippocampal gyrus and the hippocampus (Shankar *et al.*, 2008). Many research confirmed that large insoluble fibrillary aggregates and A β plaques stimulate microglia and astrocytes to produce cytotoxic proinflammatory cytokines and reactive oxygen species (ROS) that induce neuronal death (Chiarini *et al.*, 2006).

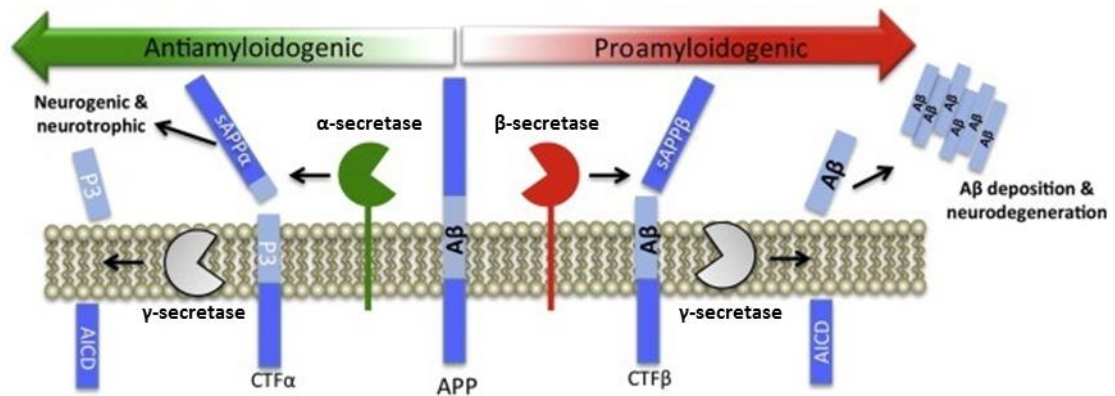


Figure 6. Scheme presenting antiamyloidogenic and amyloidogenic pathway. Processing of APP in the antiamyloidogenic pathway through α -secretase cleavage (left panel) and proamyloidogenic pathway through β -secretase cleavage (right panel). Sequential β - and γ -secretase cleavage generates neurotoxic $A\beta$ peptide species: $A\beta_{1-40}$ and $A\beta_{1-42}$ whereas APP cleaved by α - and γ -secretase leads to production of soluble sAPP α and non-amyloidogenic P3 fragment. (Adapted from Vassar, 2013).

Recent evidence demonstrate that $A\beta$ plaques are found not only extracellularly but can be present in various neuronal compartments including the Golgi apparatus, the endoplasmic reticulum, endosomes, the secretory vesicles and autophagic vacuoles (Muresan and Muresan, 2006). However, mostly the extracellular $A\beta$ regulates the anterograde axonal transport inhibition in AD and is directly involved in AD pathogenesis (Rodrigues et al., 2012). It was also shown that $A\beta$ binds the cellular prion protein (PrPc) and activates FYN inducing axon degeneration, synapse loss, memory deficits and neuronal death (Um *et al.*, 2012). FYN kinase activation increases N-methyl-D-aspartate receptor (NMDAR) expression by its phosphorylation that alters its function and leads to dendritic spine and surface receptor loss (Um *et al.*, 2012). FYN kinase associated with TAU protein form abnormal FYN-TAU interactions which sensitise synapses to glutamate excitotoxicity (Um *et al.*, 2012). This indicates the role of PrPc-FYN signalling in $A\beta$ and TAU pathologies. Downregulation of this pathway may be a potential therapeutic approach.

2.8.3 Cholinergic system dysfunction

The dysfunction and loss of bFCNs and their cortical projections are observed in the early stages of AD development. In patients with AD, the deterioration of the cognitive functions is related to the extensive neuronal loss in the basal forebrain and the loss of cholinergic neurotransmission in the cerebral cortex and another regions of the CNS. Studies on the post-mortem brains revealed a decrease in choline uptake and ACh release as well as cholinergic deficit that are associated with

the cognitive impairment and accumulation of amyloid plaques and NFTs (Nelson *et al.*, 2009). Many data from the AD brain analysis have shown a decline in the activity of ChAT and AChE, decreased ACh secretion and downregulation of nicotinic and muscarinic receptor expression (Mufson *et al.*, 2003; Parikh *et al.*, 2014). Moreover, it was reported that ACh synthesis and secretion are lowered in the presence of A β peptides (Auld *et al.*, 2002). Despite a low level of AChE in the AD brain, higher activity of this enzyme was detected in the amyloid plaques and NFTs. The increased activity of AChE was explained by an indirect effect of A β 42 mediated via α 7 nicotinic acetylcholine receptor (nAChR) subtype permeable to Ca²⁺, voltage dependent Ca²⁺ channels (Fodero *et al.*, 2004) and oxidative stress (Melo, *et al.*, 2003). Exposition to A β peptides resulting in neuronal overexcitation and increase of intracellular Ca²⁺ accumulation by stimulation of the glutamate transport in the amyloid deposition domains (Wenk, *et al.*, 2006). Molecular modelling studies indicated interactions of AChE with the A β peptides that stimulate formation of amyloid fibrils. Hydrophobic structural motifs in AChE close to the peripheral anionic binding site of the enzyme, have been shown to promote amyloid formation and its incorporation into the fibrils (De Ferrari *et al.*, 2001). Stable interactions between AChE and A β fibrils change the biochemical properties of the enzyme such as sensitivity to low pH and increase A β neurotoxicity. It may be confirmed by the fact that these complexes are more toxic for neurons than A β fibrils alone (Alvarez *et al.*, 1998). Furthermore, *in vitro* studies on transgenic Tg2576 mice revealed elevated expression of AChE in the neocortex and hippocampus, which was associated with the altered structure of the cholinergic fibers (Apelt, *et al.*, 2002). Additionally, choline uptake and muscarinic acetylcholine receptor (mAChR)/nAChR expression were significantly diminished in these mouse. Impairment of mAChR signalling pathway was also detected in APP/PSEN1 double transgenic mouse. Reduced density of mAChRs was the effect of the disruption in mAChR – G protein coupling (Machová *et al.*, 2008), which together with reduced ChAT activity are involved in AD pathogenesis.

Abnormal accumulation of A β , especially A β 42 triggers the dysfunction of cholinergic neurons by inducing aberrant cortical neuritic sprouting (Masliah *et al.*, 2003). Aberrant sprouting detected in AD brain by increase expression of growth associated protein 43 (GAP43) in response to A β leads to fiber swelling and swollen profiles arranged in a grape-like structures (Gaykema *et al.*, 1992). The appearance of swelling fibers coupled with A β fibrils block the axonal transport, which is also associated with the impairment of microtubule based neuronal transport pathways, resulting in pathological slowdown of the molecular transport processes in the progressing AD. Furthermore, the reduction of axonal transport can induce APP cleavage that results in higher amount of accumulated A β and formation of A β plaques (Stokin *et al.*,

2005). Chronic impairment of intra-axonal transport in cholinergic neurons may promote retrograde degeneration of bFCNs. It was shown that A β oligomers activate the endogenous casein kinase 2 (CSNK2) which inhibits bidirectional axonal transport involved in normal neuronal functioning (Pigino *et al.*, 2009).

Dysfunction of cholinergic system may be related with the chronic inflammatory processes observed in AD patients (Heneka *et al.*, 2015). The amyloid plaques and NFTs stimulate inflammatory reaction by activation of astrocytes and microglia, and secretion of inflammatory cytokines. A chronic increase in the levels of circulating cytokines such as interleukin 1 alpha (IL1A), interleukin 1 beta (IL1B), interleukin 6 (IL6) and tumor necrosis factor (TNF) associated with AD lesion may induce production of cytotoxic molecules and acute-phase proteins including abnormal platelet APP (Blasko *et al.*, 2000). Secretion of pro-inflammatory cytokines can promote A β synthesis that increase release of cytokines from astrocytes and microglia in a positive feedback mechanism (Gitter *et al.*, 1995). Thus, a direct link between A β -induced neurotoxicity and cytokine production has been confirmed in AD pathogenesis (Sala *et al.*, 2003).

2.8.4 GSK3 activation

Some data has shown that A β accumulated in AD brain can activate few kinases that promote TAU phosphorylation. One of the major TAU kinase involved in AD pathogenesis is GSK3B (Hernandez *et al.*, 2012) expressed in the neurons in the brain. GSK belongs to the PDPK class and its activity is controlled by phosphorylation. Phosphorylation of serine 21 in the GSK3A and serine 9 and 389 in the GSK3B isoform inactivate the kinase, whereas the activation of the enzyme depends on the phosphorylation at tyrosine 279 (GSK3A) and tyrosine 216 (GSK3B) (Wang *et al.*, 1994). The previous studies revealed the presence of 42 epitopes in TAU that can be phosphorylated by GSK3, among them 29 were detected in AD brain (Lovestone *et al.*, 1994). The most known TAU epitope phosphorylated by GSK3B is Thr231. GSK3B phosphorylates serine and threonine residues in PHF and its activity corresponds with increased A β expression and A β mediated neuronal death. Scientific evidences suggest that GSK3 is involved in many pathological hallmarks of AD including hyper-phosphorylation of TAU, increased A β production, memory impairment and neuronal loss (Cho and Johnson, 2004).

The analysis of post-mortem AD brain tissue showed an increased level of GSK3B and correlation of its activity with increasing NFT depositions (Leroy *et al.*, 2002). Overexpression of GSK3B in neurons dramatically increases the phosphorylation of TAU at various epitopes. Moreover, TAU and GSK3B have been shown to be a part of a microtubule associated TAU

phosphorylation complex which stimulate TAU modifications (Sun *et al.*, 2002). The recent studies indicated that GSK3B may be also involved in TAU degradation by the proteasomes. Inhibition of proteasome activities together with the enhanced TAU phosphorylation cause aggregation of TAU in oligodendrocytes (Goldbaum *et al.*, 2003). TAU phosphorylated by GSK3B is bound by heat shock protein family (HSP70) that activate E3 ligase carboxyl terminus of HSP70-interacting protein (CHIP) leading to TAU ubiquitination. The presence of CHIP diminishes cell death caused by TAU and GSK3B overexpression (Shimura *et al.*, 2004). Another study demonstrated that long-term treatment of neurons with insulin or IGF1 activates AKT which reduces GSK3B activity by phosphorylation at Ser9 causing decrease of TAU phosphorylation. However short-term treatment with insulin or IGF1 increases TAU phosphorylation by the activation of FYN kinase and GSK3B (Lesort and Johnson, 2000). It was also shown that increased level of intracellular calcium promotes GSK3B-mediated TAU phosphorylation. Higher GSK3B activity was confirmed by phosphorylation of Tyr216, suggesting that the transient increases in calcium activates a calcium-dependent GSK3B phosphorylation (Hartigan and Johnson, 1999).

Some evidences suggest that A β interferes with GSK3 and activates the kinase in primary hippocampal cultures whereas inhibition of GSK3B reduces A β toxicity. Although activation of two GSK isoforms was detected in APP mutant animal models, only the GSK3B promotes pathological effect on TAU (Terwel *et al.*, 2008). Activation of GSK3 by A β peptides induces pathological changes such as TAU hyperphosphorylation or impairments of mitochondrial trafficking (Amadoro *et al.*, 2011). It was shown that co-overexpression of GSK3A and APP increased the A β production, while suppression of GSK3A reduced A β level (Phiel *et al.*, 2003). Similar decrease in A β secretion was observed after deactivation of GSK3B (Serenó *et al.*, 2009).

Studies performed on cultured neurons have shown that a selective inhibitor of GSK3, lithium, significantly reduce TAU phosphorylation and protect cells against neurodegeneration (Alvarez *et al.*, 1999). Hyper-activated GSK3B disturbs proper TAU functioning through impairment of microtubule stability and their self-assembly, and dysregulation of organelle and axonal transport as reviewed in (Lei *et al.*, 2011). All the above observations provide a strong evidence that GSK3 involved in hyper-phosphorylation of TAU and A β accumulation might contribute to the pathological changes in AD brains.

2.8.5 Oxidative stress

Studies on the AD brain tissue have revealed a significant extent of oxidative damage associated with the abnormal extracellular A β plaque formation and intracellular NFTs (Zhu *et al.*, 2007). Many groups have demonstrated a crucial role of three metals: copper, iron and zinc in A β production and AD pathogenesis as reviewed in (Kozłowski *et al.*, 2012). Copper and zinc have very high affinity to bind to the N-terminal metal-binding domains located in the A β and APP (Barnham *et al.*, 2003). Moreover copper, which is present in high concentration in the A β plaques promotes generation of hydroxyl radical (the neutral form of the hydroxide ion OH⁻), one of the most reactive oxidant that contributes with increased oxidative stress in the AD brain (Strozyk *et al.*, 2009). It was indicated that high concentration of zinc found in neocortex, amygdala and hippocampus of AD brain impairs memory and cognitive functions (Deibel *et al.*, 1996). Binding of zinc to A β induces production of toxic, fibrillary aggregates leading to oxidative stress and cytotoxicity.

Biochemical analysis of AD tissues revealed many pathological changes related with ROS production. Compared to control, AD patients have higher level of malondyaldehyde and 4-hydroxynonenal in brain and cerebrospinal fluid (Lovell *et al.*, 1995). There is increased in protein carbonyl moieties in the frontal and parietal cortices and hippocampus in AD brain as well as in hydroxylated guanine level (Gandhi and Abramov, 2012). The above results are additionally confirmed by data from animal AD models in which protein and lipid peroxidation are increased in the cortex and hippocampus prior to the appearance of plaques or tangle pathology (Butterfield *et al.*, 2001). In AD patients also mitochondrial dysfunction was detected. A reduction in complex IV activity was demonstrated in mitochondria from the hippocampus and platelets of AD patients. Accumulation of A β leads to oxidative stress, mitochondrial dysfunction, and energy failure prior to the development of plaque pathology (Caspersen, 2005). A β deposits in the cells caused calcium homeostasis disturbance through increased of cytoplasmic and mitochondrial calcium level resulting in increase in ROS production and opening of the permeability transition pores (PTP) (Abramov *et al.*, 2004). Moreover, A β can directly interact with PTP component: cyclophilin D and form a complex that has reduced threshold for opening in the presence of the mitochondrial PTP (mPTP) inducers. Downregulation of cyclophilin D level by prevention of PTP opening may improve mitochondrial function in AD mouse model (Du *et al.*, 2011). In human AD brain fragmented mitochondria were localized in hippocampus. They were associated with a downregulation of mitochondrial fusion proteins: mitofusin 1 (MFN1), mitofusin 2 (MFN2), OPA1 mitochondrial

dynamins like GTPase (OPA1) and with an increase in expression of the fission, mitochondrial 1 (FIS1) protein (Du *et al.*, 2011).

One of the proposed mechanisms in the neuropathology of AD involves nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Isoforms of NADPH: NADPH oxidase 1 (NOX1), NADPH oxidase 2 (NOX2) and NADPH oxidase 3 (NOX3) were detected in the AD brain (Wilkinson and Landreth, 2006). A β activates NADPH oxidase in human phagocytes and microglia. Then, active NADPH oxidase transfers protons through the membrane and opening of an ion/anion channel is required for charge compensation. In A β activated microglia, chloride intracellular channel 1 (CLIC1) is inhibited and it decreases superoxide production, that protects cells by blocking the charge compensatory mechanism of NADPH oxidase (Milton *et al.*, 2008). Generation of NADPH oxidase in response to A β stimulation can cause damage of surrounding cells because of overproduction of nitric oxide (NO) and peroxynitrate. A β can also activate NADPH oxidase by inducing Ca²⁺ entry into astrocytes. It results in oxidative stress generation and depolarization of the mitochondrial membrane. Therefore active NADPH oxidase in combination with Ca²⁺ induce opening of the mPTP and activation of phospholipase C that change membrane structure (Abramov *et al.*, 2004). The oxidative stress signal is transferred from astrocytes to neighbouring neurons. The above mechanism is not well known although neuronal production of glutathione (GSH) – a key antioxidant, requires glutathione precursors derived from extracellular cleavage of GSH released from astrocytes. It was suggested that GSH depletion in the astrocytes due to increased oxidant production by NADPH oxidase can decrease GSH secretion from astrocytes and remove GSH in neurons (Abramov *et al.*, 2004). Nowadays there is not so many information about the direct activation of NADPH oxidase in neurons of AD models. However some studies have shown that A β and the presenilins possess the ability to activate NADPH oxidase in primary neurons (Hashimoto *et al.*, 2002).

2.8.6 Human iPSCs in the study of Alzheimer's disease

Generation of human iPSCs provides a new tool for studying the molecular basis of neurodegenerative diseases, including Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, schizophrenia or autism spectrum disorder (Corti *et al.*, 2015). An increasing number of studies have shown that iPSCs generated from patients's somatic cells could recapitulate the major cellular phenotypes of the given disease, therefore may be used for disease modelling or drug development (Boissart *et al.*, 2013). Modelling of AD with iPSCs was initiated few years ago and was based on the modelling of familial cases with well-known mutations in *APP*, *PSEN1* or *PSEN2*. Until now only few groups reported successful production

of iPSCs-derived neurons from fAD and sAD patients as shown in **Table 2**, indicating that modelling AD with iPSCs is still in the initial step.

In 2011, Yagi's group first reported generation of iPSCs from familial AD patients carrying mutations in *PSEN1* (A246E) and *PSEN2* (N141I) using retroviral transduction with OCT4, SOX2, KLF4, LIN28 and NANOG (Yagi *et al.*, 2011). After two weeks of neuronal differentiation, iPSC-derived neurons from AD patients showed an elevated level of A β 1-42 and increased A β 1-42/A β 1-40 ratio. Treatment with the Compound E and Compound W, γ -secretase inhibitors, lowered A β 1-40 and A β 1-42 levels, while only high concentration of Compound W reduced A β 1-42/A β 1-40 ratio. The lack of any form of tauopathy in the above neurons could be explained by relatively short time of neuronal maturation. Sproul *et al.* have also observed higher ratio of A β 1-42/A β 1-40 in fibroblasts, NPCs and early neurons obtained from *PSEN1* (A246E and M146L) mutants (Sproul *et al.*, 2014). According to their report, expression of 14 genes are differentially regulated in PSEN1 NPCs. Within them 5 genes such as GDNF family receptor alpha 3 (*GFRA3*), *ISL1*, *DLX1*, semaphorin 3B (*SEMA3B*) and Erb-B2 receptor tyrosine kinase 3 (*ERBB3*) displayed different expression pattern in late onset AD / intermediate AD brains. Increased secretion of A β 1-40 was also detected by Israel *et al.* in iPSC-derived neurons from two fAD patients with a duplication of APP and one LOAD patient (Israel *et al.*, 2012). However, these neurons did not display elevated ratio of A β 1-42/A β 1-40 and A β 1-38/A β 1-40. Additionally, neurons from fAD and LOAD patients had elevated phosphorylation of TAU at Thr231, increased level of active GSK3B and they accumulated large RAB5A, member RAS oncogene family (RAB5A)-positive early endosomes. Treatment of the cells with γ - and β -secretase inhibitors decreased A β 1-40 amount in one APP mutant and one sAD line, whereas only β -secretase inhibitor significantly reduced TAU phosphorylation at Thr231 and level of active GSK3B. It suggests a direct link between APP processing, but not A β in GSK3B activation and TAU phosphorylation. Muratore *et al.* described generation of iPSCs from patients with the London fAD APP mutation (V717I) and their differentiation towards forebrain neurons (Muratore *et al.*, 2014). They detected very high APP expression and A β production as well as perturbations in cleavage of APP by secretase in all APP mutant lines. β -secretase cleavage was increased although the V717I mutation was located near the γ -secretase cleavage site in the APP transmembrane domain resulting in elevated level of sAPP β and A β . In addition, APP V717I mutation affected γ -secretase cleavage leading to elevated level of A β 1-42 and A β 1-38 and a slight increase in A β 1-40 production. The altered APP processing caused also increase of total and phosphorylated TAU level. Interestingly, the treatment of early neuronal culture with A β specific antibodies decreased total TAU level, indicating altered amyloid production in the

APP mutants. It suggests that changes related with TAU level are relevant to A β phenotype and A β generation leads to increased TAU. In 2013, Kondo *et al.* successfully established iPSCs using episomal vectors from both sporadic AD and familial AD patients carrying the APP V717L and APP E693 Δ mutations (Kondo *et al.*, 2013). Compared to controls, differentiated APP V717L neurons exhibited increased extracellular level of A β 1-42 and increased A β 1-42/A β 1-40 ratio while neurons with APP E693 Δ mutation showed decreased secretion of A β 1-40 and A β 1-42. Extracellular level of secreted A β in sporadic lines was comparable with control samples. Furthermore, gene analysis of the astrocyte/neuronal co-cultures from E693 Δ neurons revealed upregulation of the oxidative stress-related genes, suggesting ER and Golgi perturbation.

Table 2. Alzheimer's disease modelling with iPSCs.

Cell type	AD models	Phenotype	Reference
fAD-iPSCs	mutations in PSEN1 (A264E), PSEN2 (N141I)	- increased A β 1-42 secretion in PSEN mutant	(Yagi <i>et al.</i> , 2011)
fAD-iPSCs sAD-iPSCs	APP duplication	- increased A β 1-40 secretion in AD-derived neurons; - increased phosphorylation at Thr231 in AD neurons; - higher level of active GSK3B in AD neurons - accumulation of RAB5A-positive endosomes in AD neurons	(Israel <i>et al.</i> , 2012)
fAD-iPSCs sAD-iPSCs	mutations in APP (V717L, E693 Δ)	- increased extracellular level of A β 1-42 and increased A β 1-42/A β 1-40 ratio in APP V717L neurons; - decreased secretion of A β 1-40 and A β 1-42 in APP E693 Δ neurons; - elevated endoplasmic reticulum and oxidative stress in APP E693 Δ neurons	(Kondo <i>et al.</i> , 2013)
fAD-iPSCs	mutation in APP (V717I)	- increased APP expression in APP mutant; - elevated level of sAPP β and A β in APP mutant; - increased level of A β 1-42 and A β 1-38 in APP mutant; - increase in levels of total and phosphorylated TAU in APP mutant	(Muratore <i>et al.</i> , 2014)
fAD-iPSCs	mutations in PSEN1 (A246E, M146L)	- higher ratio of A β 1-42/A β 1-40 in PSEN1 mutants; - different expression of 14 genes in PSEN1 mutants	(Sproul <i>et al.</i> , 2014)
sAD-iPSCs		- expression of GSK3B and pTAU in AD neurons; - changes in gene expression and inducible subunits of the proteasome complex in AD-derived neurons	(Hossini <i>et al.</i> , 2015)

Most studies used for modelling AD utilize cells with defined mutations in *PSEN* or *APP* genes. Only few groups examined cells from sporadic AD, although sAD patients represent the vast majority of AD cases. 60-80% of them may have genetic background, but only one known robust factor affecting sAD risk is *APOE* gene. The lack of defined mutation makes impossible to create isogenic controls. So far only two sAD-derived neurons generated by two groups presented disease phenotypes similar to fAD cells (Israel *et al.*, 2012; Kondo *et al.*, 2013). In 2014, Duan *et al.* generated bFCNs from iPSCs derived from normal controls, fAD and sAD

patients with APOE3/E4 genotypes (AD-E3/E4). AD-E3/E4 neurons exhibited increased A β 1-42/A β 1-40 ratio and altered response to γ -secretase inhibitor treatment compared to fAD and control lines. bFCNs from AD-E3/E4 displayed higher sensitivity to glutamate-mediated cell death, which was associated with increase intracellular Ca²⁺ level upon glutamate exposure (Duan *et al.*, 2014).

3. 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.

3.1 Rabbit stem cells and their derivatives

3.1.1 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 (mixture of Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12, 20% knock-out serum replacement (KSR), 2 mM GlutaMax, 1x Nonessential Amino Acids (NEAA), 0.1 mM β -mercaptoethanol), which was supplemented with 4 ng/ml human basic fibroblast growth factor (bFGF) and 10^3 units/ml ESGRO LIF (Merck Millipore). The media was changed daily and the cells were passaged every 3-4 days using 0.05% trypsin- EDTA.

3.1.2 Immunocytochemistry and AP staining

Cells were fixed in 4% paraformaldehyde (PFA) for 30 min at RT, then permeabilized in PBS containing 0.1% Triton X-100 for 5 min and blocked with 3% bovine serum albumin (BSA) for 60 min at RT. The cells were incubated with primary antibodies overnight at 4°C (**Supplementary Table 1**). To visualize the signal the following secondary antibodies were applied: Alexa Fluor 488 donkey anti-mouse IgG (H+L) and Alexa Fluor 594 donkey anti-rabbit IgG (H+L) (Life Technologies). For nuclei counterstaining cells were incubated 20 min at RT with 0.2 μ g/ml 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).

3.1.3 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 β -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.

3.1.4 Dual SMAD inhibition on rbiPSCs

The dual inhibition of SMAD signaling pathway using LDN193189 and SB431542 (Chambers *et al.*, 2009a) 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 (NIM) (1:1 (v/v) mixture of Dulbecco's Modified Eagle's/F12 and Neurobasal Medium, 1x N-2 Supplement, 1x B-27 Supplement, 1x NEAA, 2 mM L-Glutamine, 50 U/ml Penicillin/Streptomycin, 100 μ M β -mercaptoethanol, 5 μ g/ml insulin), which was supplemented with, 5 ng/ml bFGF, 0.1 μ M or 0.2 μ M or 0.4 μ M LDN193189 (Selleckchem) and 5 μ M or 10 μ M 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) (1:1 (v/v) mixture of Dulbecco's Modified Eagle's/F12 and Neurobasal Medium, 1x N-2 Supplement, 1x B-27 Supplement, 1x NEAA, 2 mM L-Glutamine, 50 U/ml Penicillin/Streptomycin), and supplemented with 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml bFGF.

3.2 Human stem cells and their derivatives

3.2.1 Human iPSC lines

Alzheimer's disease patients derived iPSC lines used in this study were characterized and published earlier as detailed in **Table 3**. The patients were clinically diagnosed and characterized by the Institute of Genomic Medicine and Rare Disorders, Semmelweis University, Budapest (Hungary) or at the Danish Dementia Research Centre, Rigshospitalet, University of Copenhagen according to the national neurology examination standards. Hungarian patient derived iPSC lines were generated by reprogramming of peripheral blood mononuclear cells (PBMCs), isolated from patients' peripheral whole blood samples, while in Denmark, skin tissue biopsies were collected and fibroblast cells were isolated and reprogrammed by episomal vectors. Non-dement volunteers (assessed by clinical evaluation) 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.

Table 3. Human iPSClines used in this study.

iPSC line	Clone number	Identifier	Disease	Mutation	Sex	Reference
BIOT-7183-PSEN1	S1	fAD-1	early onset	PSEN1 c.265G>C, p.V89L	F	Nemes <i>et al.</i> , 2016
	S2	fAD-2	fAD			
H234	C5	fAD-3	early onset fAD	PSEN1 c.449T>C, p.L150P	M	Tubsuwan <i>et al.</i> , 2016
H235	C6	fAD-4	early onset fAD	PSEN1 c.449T>C, p.L150P	M	-
BIOT-0904-LOAD	S2	sAD-1	late-onset	unknown	M	Táncos <i>et al.</i> , 2016b
	S3	sAD-2	sAD			
BIOT-0630-LOAD	S4	sAD-3	late-onset sAD	unknown	F	Táncos <i>et al.</i> , 2016a
BIOT-4828-LOAD	S1	sAD-4	late-onset	unknown	F	Ochalek <i>et al.</i> , 2016
	S6	sAD-5	sAD			
BIOT-0726-LOAD	S3	sAD-6	late-onset sAD	unknown	F	Chandrasekaran <i>et al.</i> , 2016
CTRL1	S9	ctrl-1	healthy	-	F	-
	S11	ctrl-2				
H250	C16	ctrl-3	healthy	-	F	-
H256	C6	ctrl-4	healthy	-	M	-

3.2.2 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 (Chambers *et al.*, 2009a). 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 μ M LDN193189 (Selleckchem) and 10 μ M 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.

3.2.3 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) (1:1 (v/v) mixture of Dulbecco's Modified Eagle's/F12 and Neurobasal-A medium, 1x N-2 Supplement, 1x B-27 Supplement, 1x NEAA, 2mM L-Glutamine, 50 U/ml Penicillin/Streptomycin), supplemented with 0.2 mM ascorbic acid (AA) (Sigma-Aldrich) and 25 μ M β -mercaptoethanol. For terminal differentiation into cortical neurons, the cells were plated on POL/L (0.002%/2 μ g/cm²) at a seeding density of 40.000 cells/cm² for ICC and 100.000 cells/cm² for ELISA and WB experiments with NMM medium. The medium was changed every 3-4 days during the course of the terminal differentiation. 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.

3.2.4 Immunocytochemistry (ICC)

Cells were fixed in 4% PFA for 20 min at RT, washed 2x with phosphate buffered saline (PBS) and permeabilized with 0.2% Triton X-100 in PBS for 20 min. Then, cells were blocked with 3% BSA in the presence of 0.2% Triton X-100 in PBS for 60 min at RT. The respective primary antibodies were applied overnight at 4°C (**Supplementary Table 1**). To detect the signal, cells were incubated 60 min at RT with the following secondary antibodies: Alexa Fluor 488 donkey anti-rabbit IgG (H+L), Alexa Fluor 594 donkey anti-mouse IgG (H+L), Alexa Fluor 488 donkey anti-mouse IgG (H+L) and Alexa Fluor 594 donkey anti-rabbit IgG (H+L) (Life Technologies). Cell nuclei were visualized using Vectashield Mounting Medium with DAPI (1.5 µg/ml; Vector Laboratories). Cells were analyzed under fluorescent microscope equipped with 3D imaging module (Axio Imager system with ApoTome; Carl Zeiss MicroImaging GmbH) controlled by AxioVision 4.8.1 software (Carl Zeiss).

3.2.5 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 CaCl₂ 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 (White *et al.*, 1979) 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).

3.2.6 Flow cytometry

NPCs growing on POL/L coated dishes were dissociated into single cells with Accutase and fixed with 4% PFA for 20 min at RT. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at RT and blocked with 10% Fetal Bovine Serum (FBS) in PBS for 20 min at RT. 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).

3.2.7 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). The protein extracts were derived from one well of a six well plate of a single neuronal differentiation. 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 (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1 % Tween-20) containing 5% BSA and then incubated with the respective primary antibody solution overnight at 4°C (**Supplementary Table 1**). After washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at RT. All the secondary antibodies were either goat anti-mouse or goat anti-rabbit purchased from Sigma-Aldrich. All samples were analyzed at day 0, 14, 28, 42, 56 and 70 of terminal differentiation and the amount of pTAU relative to total TAU (pTAU/TAU), APP and APP-CTF levels were measured. To determine the level of GSK3B activation, the proportion of active GSK3B was calculated by measuring the amount of GSK3B lacking phosphorylation at Ser9 relative to total GSKβB level. (phosphorylation at Ser9 inactivates GSK3B). 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 out using Image Studio™ Lite software (LI-COR).

3.2.8 Measurement of A β 1-40 and A β 1-42 by ELISA

Conditioned media was collected after 4 days of culture (without media change) at every week from one well of a six well plate. To prevent protein degradation, 4-(2-aminomethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) was added to the media. Extracellular A β 1-40 and A β 1-42 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. The secreted A β levels determined in pM 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 (\pm SEM) of the four healthy individual derived clones (ctrl-1, ctrl-2, ctrl-3, ctrl-4) in all experiments.

3.2.9 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 μ g of RNA was transcribed using the SuperScriptTM III VILOTM 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 μ L 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 detailed in **Supplementary Table 2**. Human *GAPDH* was used as reference gene. The data was analyzed using REST software (2009 V2.0.13).

3.2.10 Cell viability assay after H₂O₂ and A β 1-42 treatment

Neurons growing on 96 well plate, at day 28 and 56 of terminal differentiation were treated with 30 μ M and 60 μ M of hydrogen peroxide (H₂O₂) (Sigma-Aldrich) or 5 μ M A β 1-42 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 H₂O₂ and A β 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.

3.2.11 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 \pm 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 * $p < 0.05$

4. RESULTS

4.1 Rabbit iPSCs and their neuronal differentiation

4.1.1 Characterisation of rabbit induced pluripotent stem cells

Rabbit iPSCs (rbiPSCs) newly generated from embryonic fibroblasts (Tancos *et al.*, 2017) 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 (**Figure 7**).

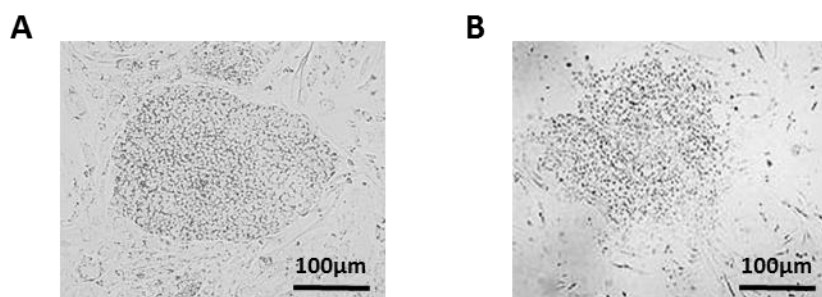


Figure 7. Morphology of rabbit iPSC colonies (rbiPSCs) (A) in comparison with the morphology of control rabbit ES colonies (rbESCs) (B). Pictures provided by Dr Zsuzsanna Táncos.

To monitor stem cell property of rbiPSCs alkaline phosphatase (AP) staining was performed. AP activity was detected in the rbiPSCs and rbESCs used as a positive control (**Figure 8A**). The newly established iPSC line was further expanded and examined for the expression of pluripotent markers using stem cell specific antibodies. Comparable to rbESCs, rbiPSCs at low passage number ($p < 10$) as well as rbiPSCs after long-term culture ($p > 20$) highly expressed OCT4 and SOX2, and the membrane surface marker SSEA4. Both rbiPSCs and control rbESCs have not shown expression of another marker SSEA1 (**Figure 8B**). These rbiPSCs upon injection into SCID mouse during the *in vivo* differentiation assay formed teratoma. The hematoxylin and eosin stain (H&E) of the isolated teratoma revealed the presence of three germ layers: ectoderm, mesoderm and endoderm (Tancos *et al.*, 2017).

The *in vitro* spontaneous differentiation of rbiPSCs led to formation of three germinal layers confirmed by the expression of ectodermal (TUBB3), mesodermal (SMOOTH MUSCLE ACTIN, S.M. Actin) and endodermal (GATA binding protein 4, GATA4) markers as well as neuroectoderm (glial fibrillary acidic protein, GFAP) marker (**Figure 9**). RbiPSCs plated at high passage number ($p > 20$) differentiated into more mature cells, with higher expression of TUBB3, S.M.Actin, GATA4 and GFAP, and displayed similar expression pattern as control rbESCs. Differentiation potential of rbiPSCs at low passage number ($p < 10$) was decreased, the cells

showed lower expression of S.M.Actin and GATA, whereas TUBB3 and GFAP expression was comparable to rbESCs.

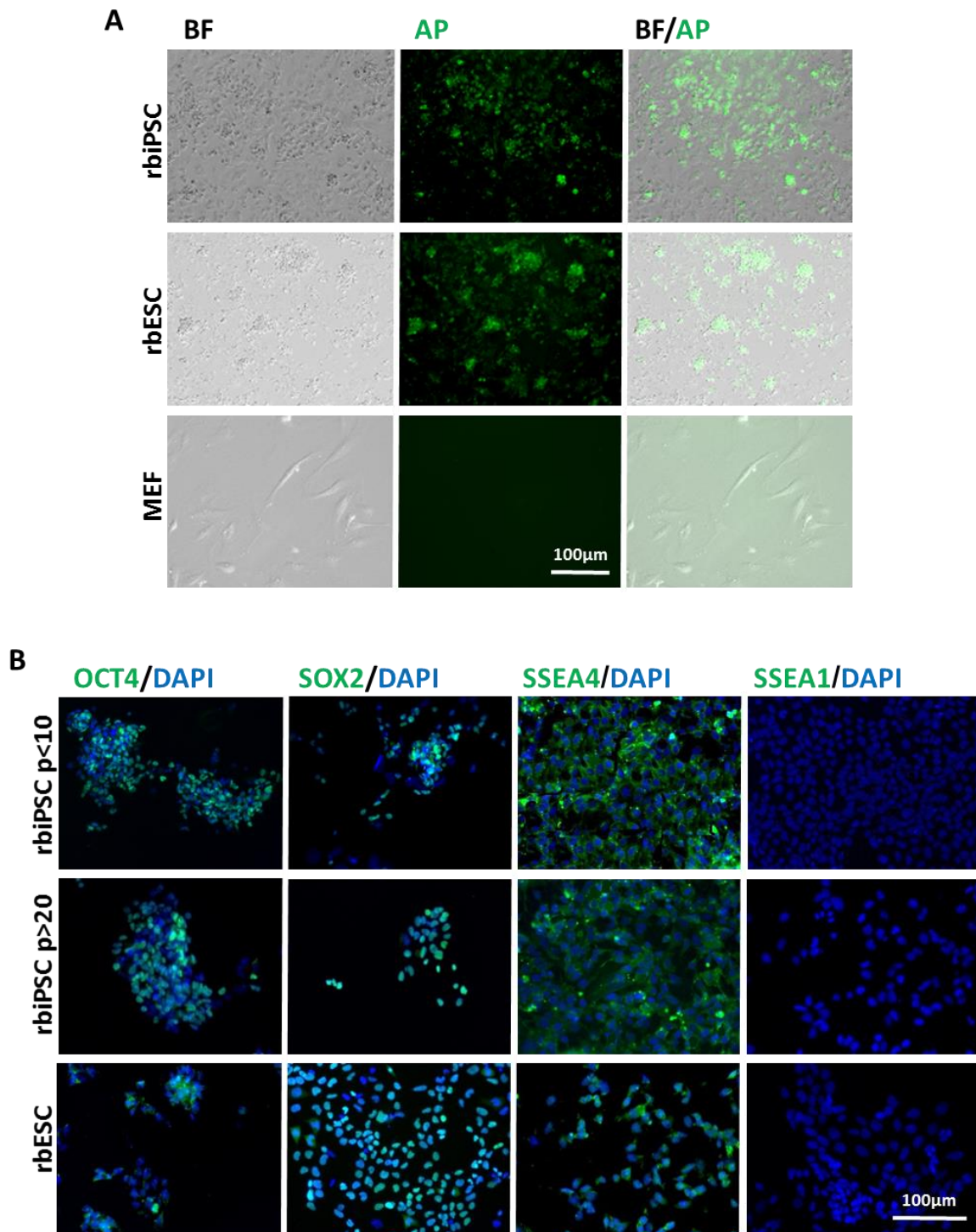


Figure 8. Characterization of rbiPSCs. (A) Alkaline phosphatase staining (AP). The expanded iPSCs show AP activity, a characteristic pluripotency marker. BF (bright field). (B) The expression of pluripotency markers: OCT4, SOX2 and SSEA4 detected in rbiPSCs with low (upper panel) and high passage number (middle panel) and control rbESCs (lower panel). SSEA1 was not expressed in rbiPSCs and control rbESCs. DAPI was used to visualize the nuclei. Scale bar: 100 μ m. Pictures provided by Dr Zsuzsanna Tánkos.

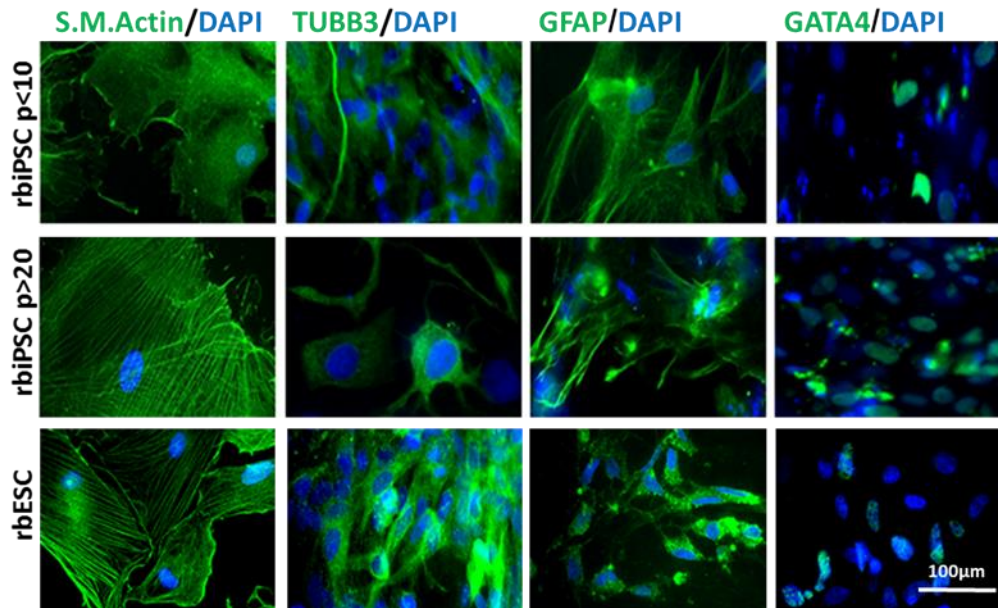


Figure 9. *In vitro* spontaneous differentiation of rbiPSCs. Comparable to control rbESCs (lower panel), rbiPSCs at low passage number ($p < 10$) (upper panel) and at high passage number ($p > 20$) (middle panel) have shown expression of S.M.Actin (mesoderm marker), TUBB3 (ectoderm marker), GFAP (neuroectoderm marker) and GATA4 (endoderm marker). Scale bar: 100 μm . Pictures provided by Dr Zsuzsanna Tánkos.

4.1.2 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. Stem cells originated from hanging drop culture formed small uniform-sized spherical EBs, while iPSCs maintained in suspension aggregated into heterogeneous EBs with different sizes (**Figure 10A**). 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 (**Figure 10B**). Furthermore, rbiPSC-derived neurons presented very short neurites and they were not able to form dense neuronal network. Upon few days of culture in differentiation conditions, the neurons stop to grow or died.

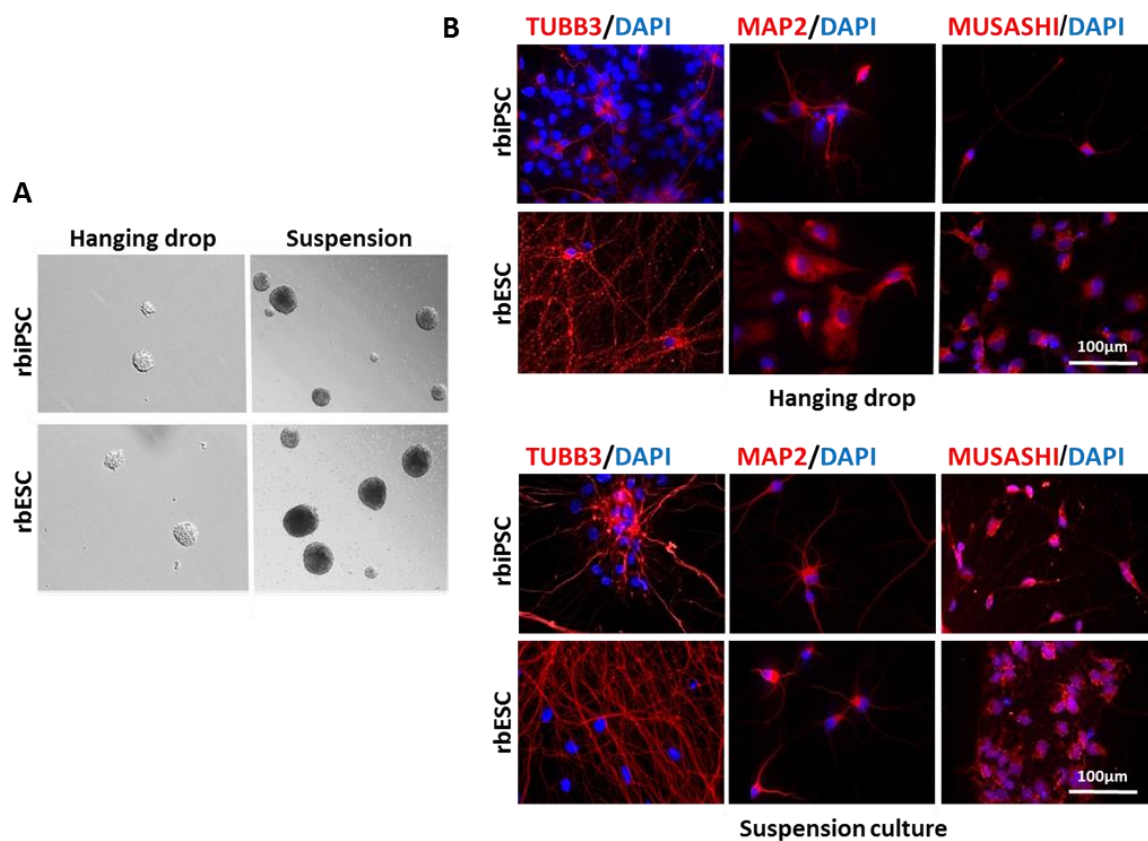


Figure 10. *In vitro* directed differentiation of rbiPSCs towards neuronal cells. (A) Comparison of embryoid bodies (EBs) generated in hanging drop and suspension cultures. (B) Expression of neural lineage markers: TUBB3, MAP2 and MUSASHI in cells originated from hanging drop (upper panel) and suspension culture method (lower panel). RbESCs were used as a control. Scale bar: 100 μm .

4.1.3 Dual SMAD inhibition of rbiPSCs

After the failure of long-term culture of EB-based differentiation derived neuronal cells we investigated if the well-known neuronal human iPSC differentiation, the dual inhibition of SMAD signalling is more effective on rabbit iPSCs than the EB based method.

Therefore, we applied the standard human protocol on rabbit iPSCs. During the course of 11 day induction we observed formation of few neural rosettes, in line with massive cell death. Therefore, we decided to investigate if a longer period of induction and different concentration combinations of the inhibitors will enhance the differentiation and result more rosette-like structures with neuronal progenitor cells. We tested 0.1 μM ; 0.2 μM and 0.4 μM LDN193189 and 5 μM or 10 μM SB431542 combinations. In our experiments 0.2 μM LDN193189 and 10 μM SB431542 combination provided the best outcome and the highest rosette-like structure formation. However, none of the tested conditions were effective to induce neuronal rosette formation with high frequency and proper quality. The number of formed rosette-like structures remained low: only one or two small rosette-like structures appeared with very low cell number

(5 up to 15 cells), which made the cultures unpropagable. During the manipulation of these rosette-like structures the cell clusters should be isolated manually, however the very loose structure and low cell number resulted the loss of these clusters during the manipulations.

After 15 days of induction we passaged the cells and replated them to POL/L surface in NIM media to enhance NPC proliferation if NPCs are present. Unfortunately, the cells were not able to attach and we could not maintain those few cells which could survive the replating. The efficiency of terminal differentiation forming neurons from rbiPSCs was very low. Furthermore, newly differentiated neurons presented very rapid and dramatic cell death and few days after differentiation almost all cells were lost. Although we repeated the experiments, we should conclude that the generation of neuronal stem cells or progenitors from rabbit iPSCs is more complex and requires further investigations than we expected. Thus, we decided to use only human iPSCs for production of neurons and building up the *in vitro* neurodegenerative disease model.

4.2 Human iPSCs and their neuronal differentiation

4.2.1 Generation of human iPSCs

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 (**Table 3**). Here, three fAD patients with mutation in *PSEN1* (contributing with in total 4 lines: fAD-1, fAD-2, fAD-3, fAD-4), four patients with late onset sAD (contributing with in total 6 lines: sAD-1, sAD-2, sAD-3, sAD-4, sAD-5, sAD-6) and three non-dement individuals as controls' iPSC clones (contributing with in total 4 lines: ctrl-1, ctrl-2, ctrl-3, ctrl-4) were studied (see also **Table 3**). In all sAD samples, direct sequencing analysis revealed the lack of pathogenic mutation in *APP*, *PSEN1* and *PSEN2*, indicating late onset AD. Moreover, the direct sequencing of *MAPT* and granulin precursor (*GRN*) excluded frontotemporal dementia as well. The APOE status of the cell lines included in the study is showed in **Table 4**.

4.2.2 Generation and characterization of human NPCs

All AD-iPSCs and control iPSCs were successfully converted into neural progenitor cells. The induction process was facilitated by inhibition of BMP4 and TGF β signalling mediated through SMADs using small molecule inhibitors: LDN193189 and SB431542. In the first phase of induction, the neuroepithelial sheets (resembling the neural plate *in vivo*) were formed and then the neural rosettes (resembling the neural tube stage *in vivo*) developed. Schematic diagram

presents the time scale and used reagents for generation of neuronal lineage from hiPSCs (**Figure 11**). All AD-derived and control iPSC lines were effectively induced into NPCs (**Figure 12**) and expressed neuroepithelial markers: PAX6 and NES (**Figure 13**). To quantify the efficiency of NPC formation flow cytometry was applied, which showed that there was no significant differences between any of the cell lines with respect to generating NPCs including more than 70% co-positivity for PAX6 and NES (**Figure 14**).

Table 4. Polymorphism of AD and FTD related genes and APOE status of cell lines used in this study. Footnote: Cell lines not detailed in the table were not permitted to be involved in whole genome sequencing, therefore only the clinical mutation identification was available.

Subject	Protein Change			APOE allele
	Gene	Disease causing mutation	Polymorphisms	
BIOT-7183-PSEN1	PSEN1	V89L	-	3/3
	PSEN2	-	A23A; N43N; H87H	
	MAPT	-	P118P; P160P; A169A; P202L; D285N; V289A; R370W; S447P; T504T; T591C;	
BIOT-4828-LOAD	PSEN1	-	E318G	3/4
	PSEN2	-	A23A; N43N; H87H	
	MAPT	-	Q230R; P212P	
BIOT-0726-LOAD	PSEN2	-	A23A; N43N; H87H	3/3
	MAPT	-	P160P	
BIOT-0630-LOAD	PSEN2	-	A23A; N43N; H87H	3/3
BIOT-0904-LOAD	PSEN2	-	N14K, N43N; H87H	3/3
	GRN	-	R433W	
CTRL1	PSEN1	-	E339E	3/3
	PSEN2	-	A23A; N43N; H87H	
	MAPT	-	D58D	
	GRN	-	D128D	

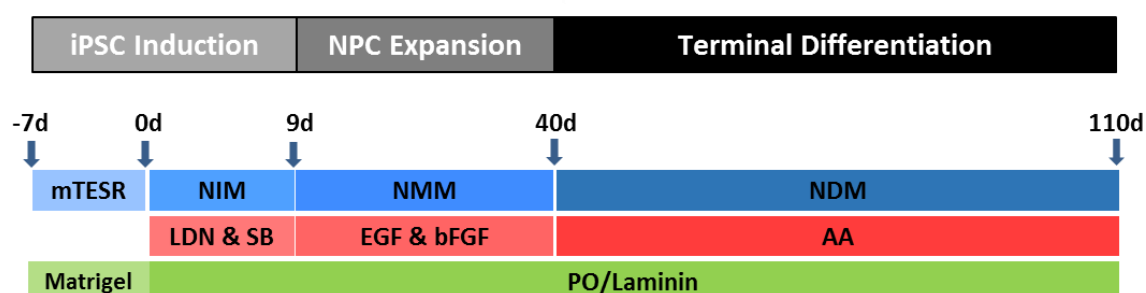


Figure 11. Scheme presenting generation of neurons from iPSC-derived neuronal progenitor cells (NPCs). iPSCs induction was performed in Neural Induction Medium (NIM) in the presence of small inhibitors of SMAD pathway: LDN and SB431542. Generated NPCs were cultured and expanded in Neural Maintenance Medium (NMM) supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). At day 40 (40d) culture media was switched to Neural Differentiation Medium (NDM) and in the presence of ascorbic acid (AA) the NPCs differentiated into neuronal cells.

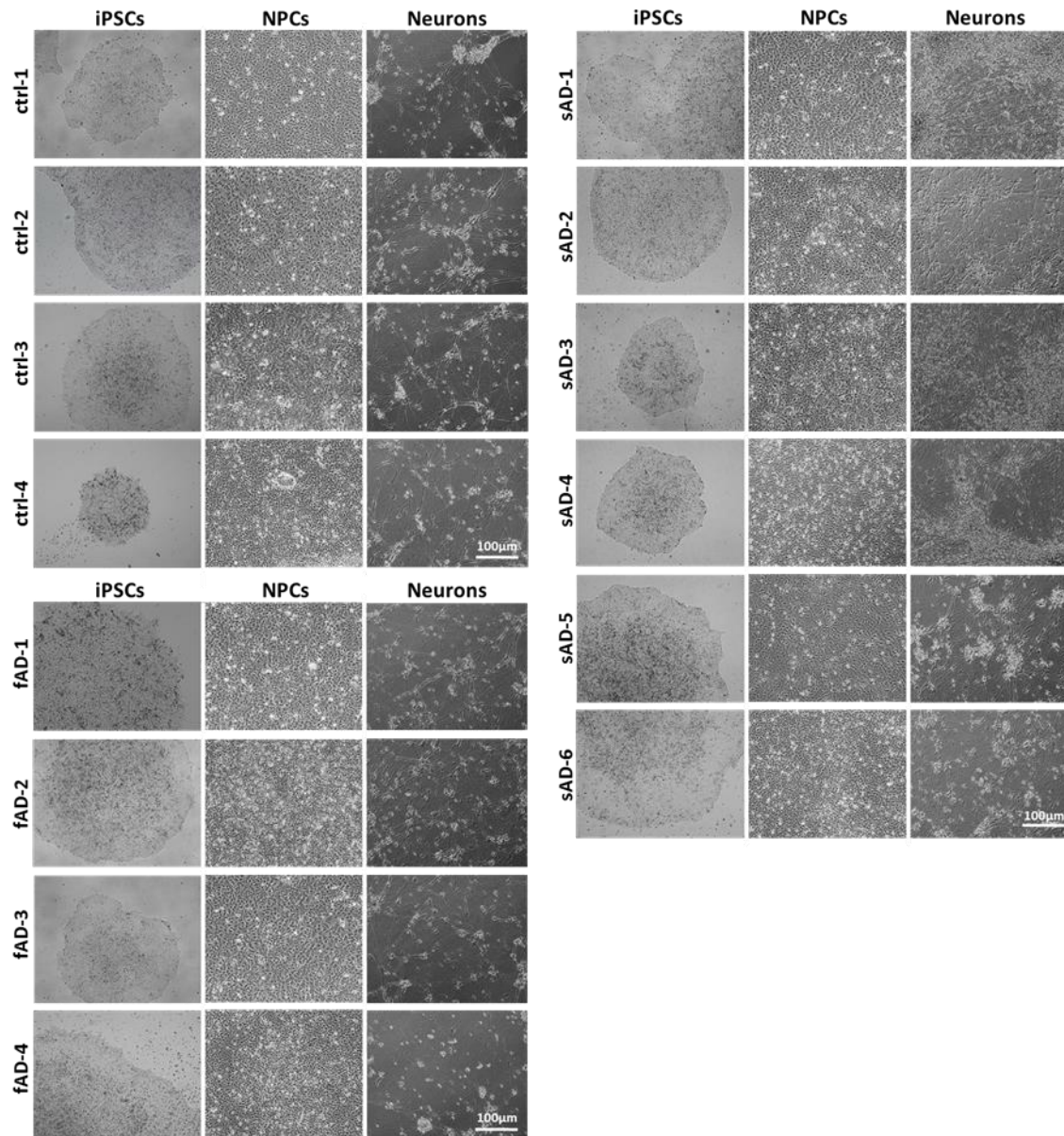


Figure 12. Bright field images presenting the morphology of human induced pluripotent stem cells (iPSCs), human neural progenitor cells (NPCs) and iPSC-derived neurons. Cells originated from control individuals (ctrl-1 – ctrl-4), and patients with familial AD (fAD-1 – fAD-4) and sporadic AD (sAD-1 – sAD-6). The images present similar morphology of iPSCs, NPCs and neurons in control, fAD and sAD cell lines. Scale bar: 100 µm.

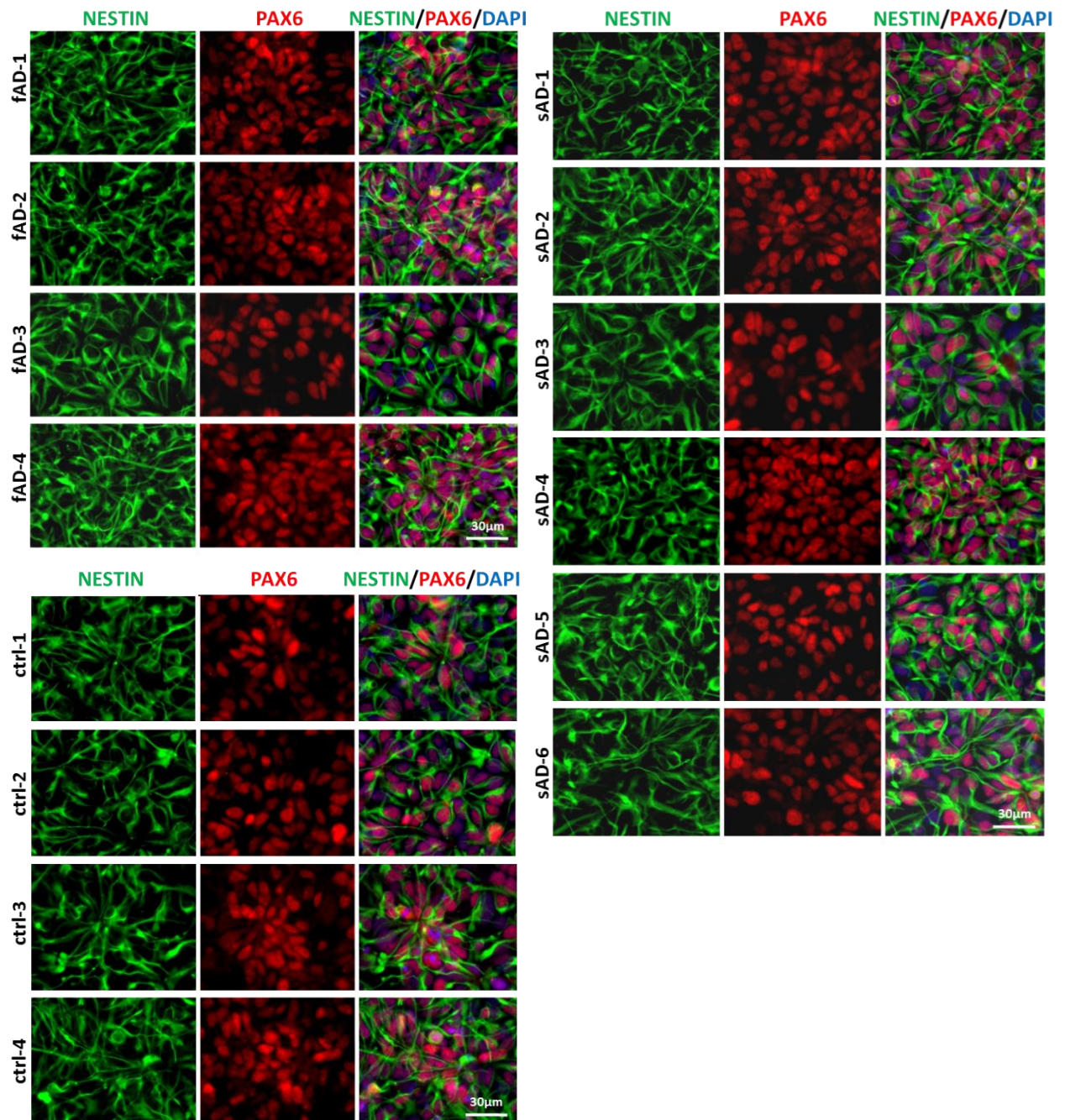


Figure 13. Immunofluorescence characterization of the iPSC-derived neural progenitor cells. Representative images of NPCs from healthy controls (ctrl-1 – ctrl-4), patients with early onset familial AD (fAD-1 – fAD-4) and patients with late onset sporadic AD (sAD-1 – sAD-6) stained with NESTIN (green), PAX6 (red) and DAPI (blue). All three groups exhibited similar progenitor marker expression. Scale bar: 30 μm.

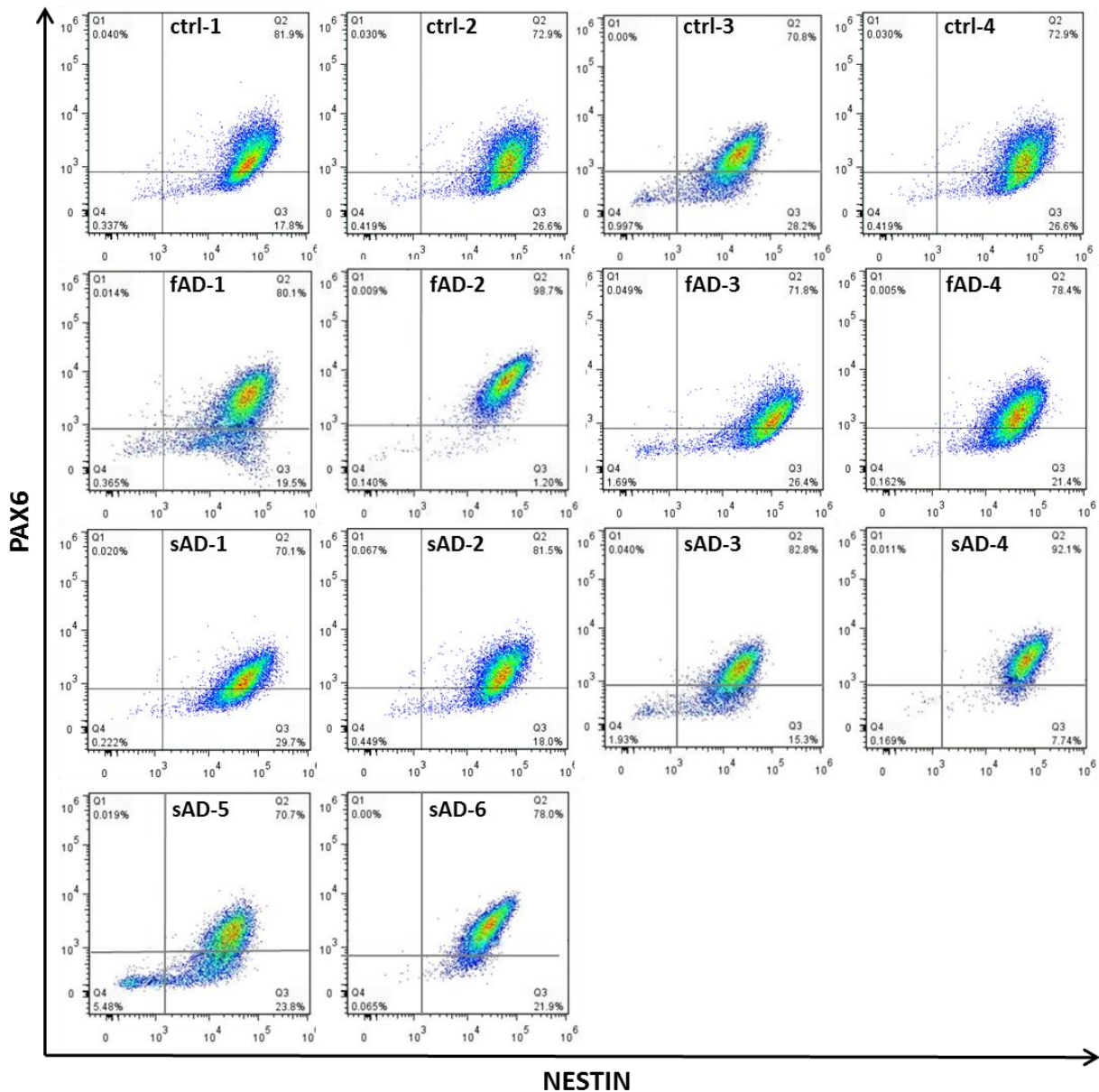


Figure 14. Flow cytometry analysis of cultured NPCs. Dot plots demonstrate similar expression pattern of PAX6 and NESTIN in control, fAD and sAD iPSC line -derived NPC cultures with similar passage numbers (p6).

4.2.3 Neuronal differentiation capacity of fAD and sAD patient derived iPSCs

The neuronal differentiation (terminal differentiation; TD) of fAD and sAD origin NPCs were investigated and compared with control lines at two different time points (TD28, TD70). Immunofluorescent labeling has shown the increasing expression of neuronal markers: TUBB3 and MAP2 during the neuronal differentiation, which was comparable among the disease and control cell lines (**Figure 15**). More mature markers, TAU (**Figure 16**) and Neurofilament, Heavy Polypeptide 200kDa (NF200) (**Figure 17**), appeared later compared to MAP2 and TUBB3.

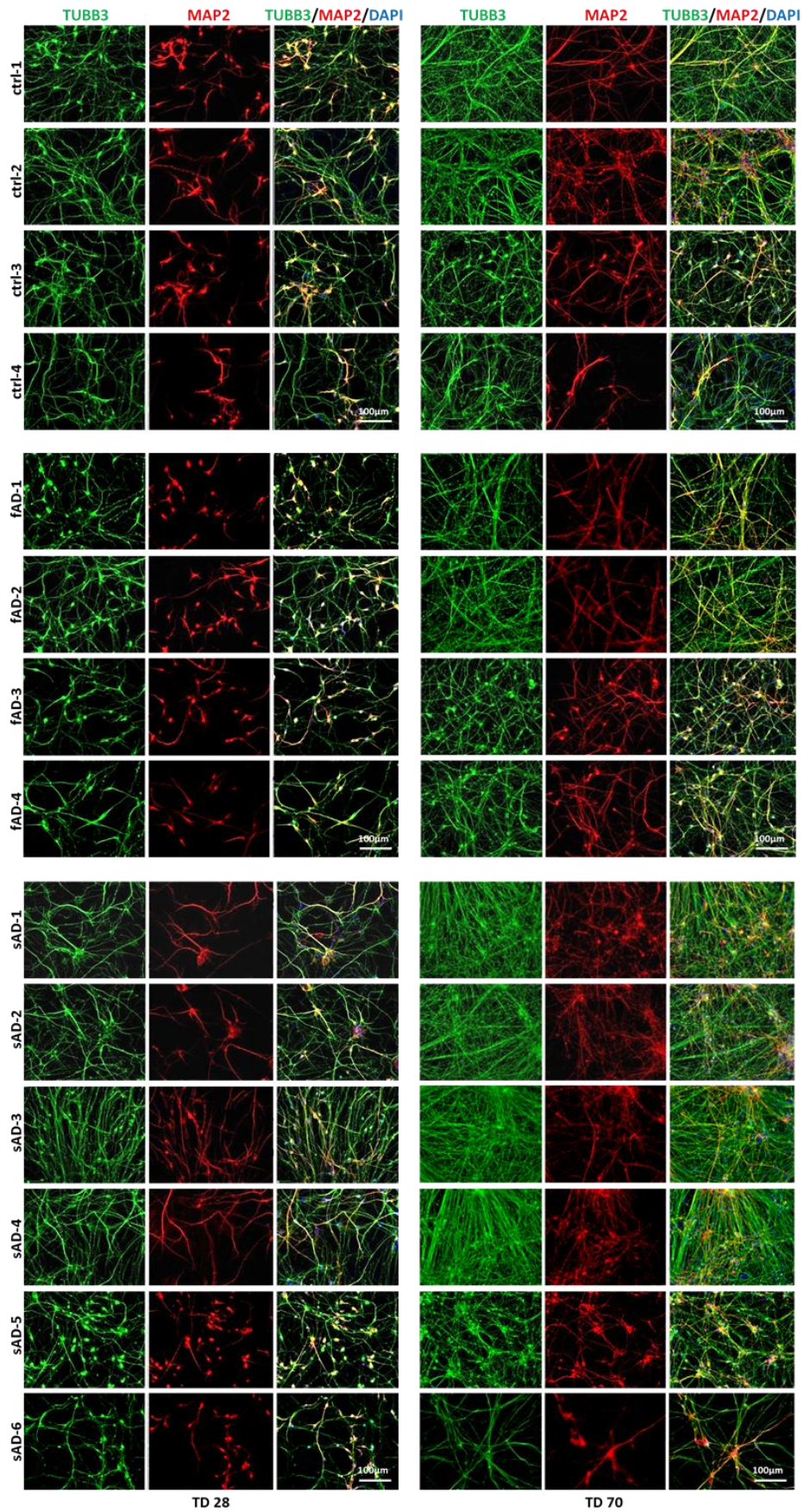


Figure 15. Neuronal differentiation of control and AD-iPSCs. Representative immunofluorescent images show expression of neuronal markers in control (ctrl-1 – ctrl-4), fAD (fAD-1 – fAD-4) and sAD- (sAD-1 – sAD-6) –iPSC -derived neurons at day 28 and 70 of terminal differentiation (TD28, TD70): TUBB3 (green), MAP2 (red). Scale bar: 100 μ m.

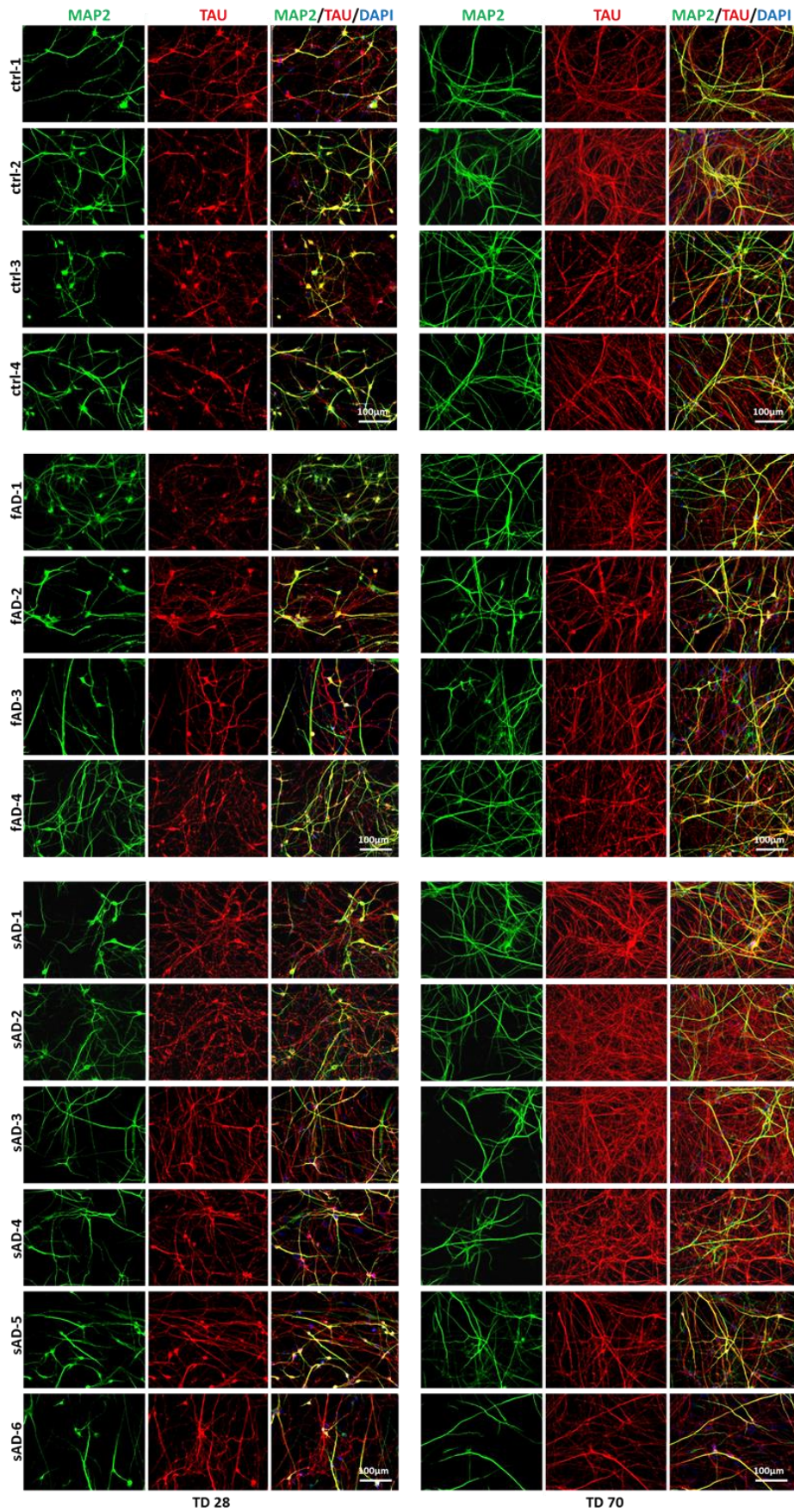


Figure 16. Neuronal differentiation of control and AD-iPSCs. Representative immunofluorescent images show expression of neuronal markers in control (ctrl-1 – ctrl-4), fAD (fAD-1 – fAD-4) and sAD- (sAD-1 – sAD-6) -iPSC -derived neurons at day 28 and 70 of terminal differentiation (TD28, TD70): MAP2 (green), TAU (red). Scale bar: 100 μ m.

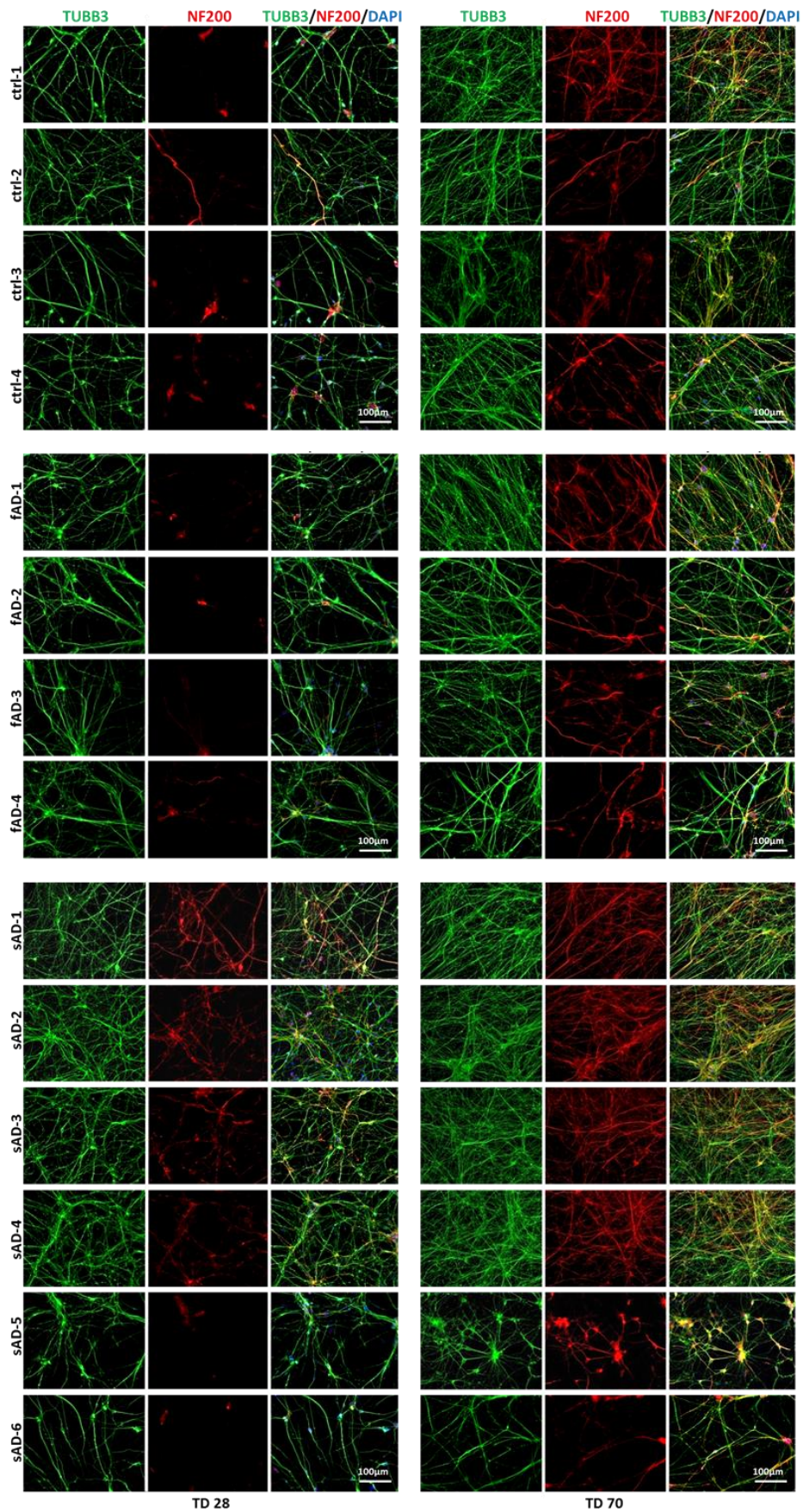


Figure 17. Neuronal differentiation of control and AD-iPSCs. Representative immunofluorescent images show expression of neuronal markers in control (ctrl-1 – ctrl-4), fAD (fAD-1 – fAD-4) and sAD- (sAD-1 – sAD-6) -iPSC -derived neurons at day 28 and 70 of terminal differentiation (TD28, TD70): NF200 (green), TUBB3 (red). Scale bar: 100 μm.

ICC analysis at day 70 of AD-derived and control neuronal cultures revealed the presence of proteins related to distinct subtypes of mature neurons: markers of cholinergic neurons (VACHT), dopaminergic neurons (TH), GABAergic neurons (glutamic acid decarboxylase 2 and -1, GAD2/GAD1; gamma-aminobutyric acid (GABA) neurotransmitter) and glutamatergic neurons (vesicular glutamate transporter 1/2; VGLUT1/2) (**Figure 18A**). Electron microscopy analysis of cell culture at TD35 revealed the presence of synapses with synaptic vesicles and synaptic cleft with synaptic junctions as well as docking synaptic vesicles (**Figure 18B**).

In order to determine gene expression levels, qRT-PCR was performed on day 70 neurons. The analysis revealed that the cells expressed high level of *MAP2*, solute carrier family 6 member 4 (*SLC6A4*), NMDA receptor subunit NR1 (Glutamate Ionotropic Receptor NMDA Type Subunit 1; *GRINI*), marker of maturing neurons – RNA binding protein, Fox-1 homolog 3 (*RBFox3*) and *GAD1* (**Figure 19**). Moreover, they also showed high expression level of dopaminergic neuron marker (*TH*), astrocyte marker (*GFAP*) and an early oligodendrocyte marker (claudin 11; (*CLDN11*). Furthermore, the cells expressed *TUBB3*, *CHAT* and postsynaptic density protein 95 (discs large MAGUK scaffold protein 4; *DLG4*). Importantly, no evident differences in the gene expression level were observed among control, fAD and sAD cell lines. In conclusion, these findings indicate that AD cell origin has no significant effect on the overall neuronal differentiation of iPSCs in the used system, and NPCs can be differentiated into various neuronal cell types with similar efficiency from the diseased cells and healthy individuals.

4.2.4 Secretion of A β by fAD and sAD iPSC-derived neurons

Upregulation of A β production is one of the main pathological hallmark of AD. To determine A β production from fAD and sAD patients' iPSC lines, secreted A β 1-40 and A β 1-42 levels were measured by ELISA in the conditioned media of differentiated neural cell lines weekly, from the second to tenth week of differentiation. Elevated secreted A β 1-40 and highly increased A β 1-42 levels were observed in all fAD neural cultures compare to mean level from the control lines at almost all time points (**Figure 20, 21, 22**). The A β 1-42/A β 1-40 ratio was also increased up to 2-fold for the fAD-iPSCs-derived neurons. Extracellular level of A β 1-40 in all sAD neurons was significantly higher compared to control and fAD neurons. The A β 1-42 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 A β 1-42/A β 1-40 ratio in all sAD-iPSCs-derived neurons was similar to those in controls (**Figure 20C, 21C**). In non-mature neurons up to day 14 (TD14) the A β production was comparable among AD and control lines (**Figure 20**).

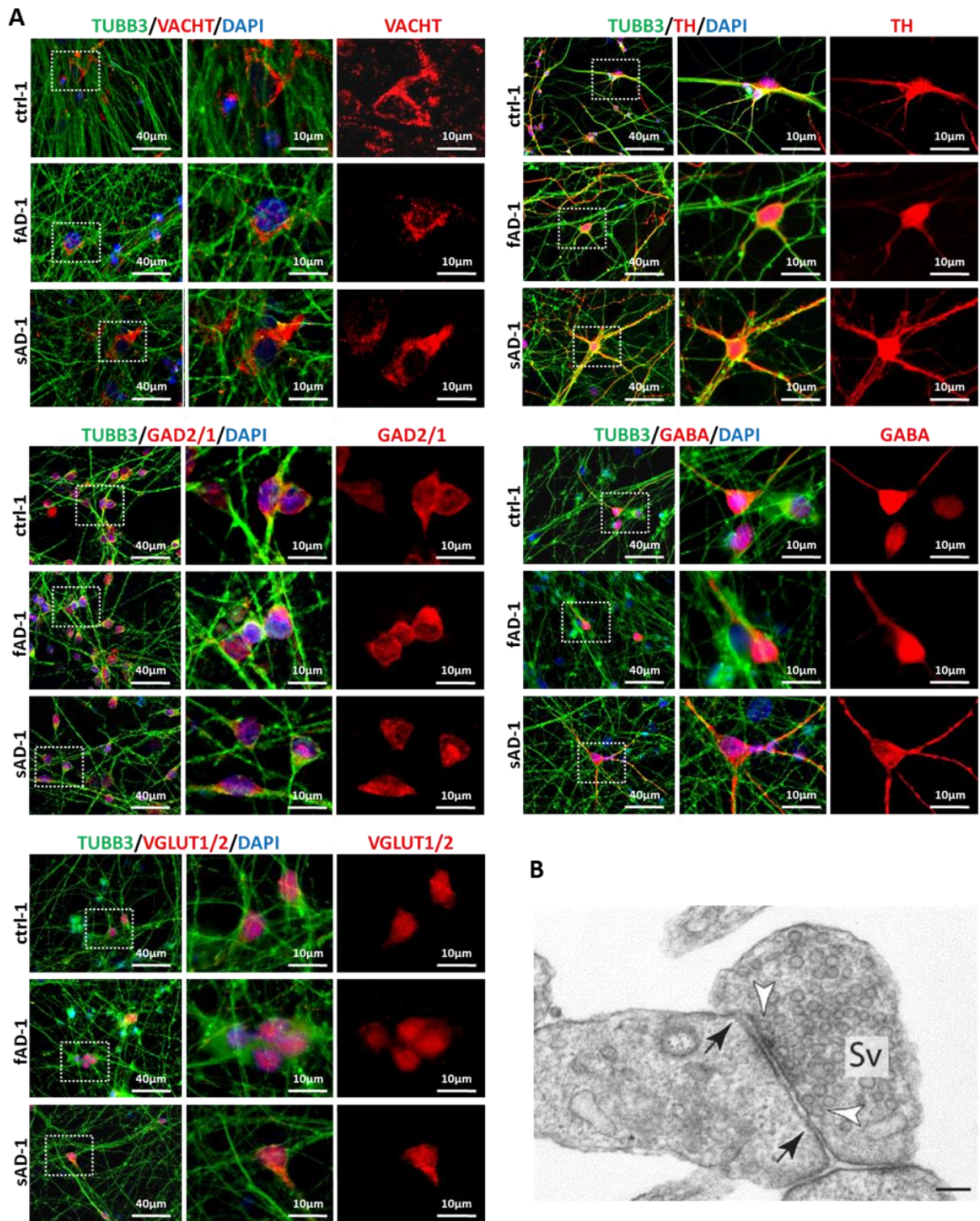


Figure 18. Neuronal differentiation of control and AD-iPSCs into various neuronal subtypes at TD70. (A) Differentiation of iPSCs was confirmed by presence of specific markers: VACHT (cholinergic neurons), TH (dopaminergic neurons), GAD2/1 and GABA (GABAergic neurons), VGLUT1/2 (glutamatergic neurons). The second and the third column in each panel are the magnification of the area marked in a white rectangular in the first column. Scale bar: 40 μm and 10 μm as indicated. (B) Ultrastructure of a synapse from ctrl-1 neurons at TD35 with synaptic vesicles (Sv) and synaptic cleft with synaptic junctions (tight junctions, black arrowheads). Docking synaptic vesicles are also observable (white arrowheads). Scale bar: 100 nm.

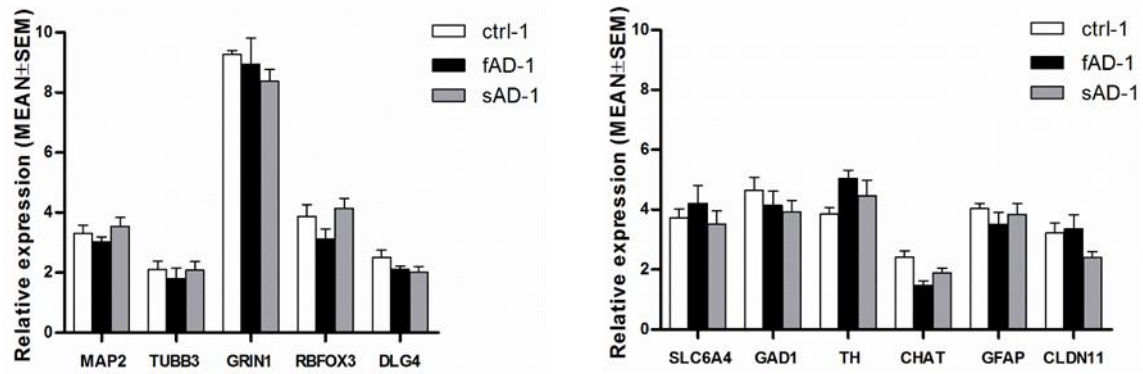


Figure 19. Gene expression of neuronal markers from ctrl-1, fAD-1 and sAD-1 lines at TD70 obtained by qPCR. The expression values were normalized to *GAPDH* and calculated as a relative amount of mRNA versus expression values of NPCs of each cell line, which was set to 1. Data was reported as MEAN \pm SEM of 3 independent measurements. AD-iPSC neurons demonstrate similar expression fashion as neuronal cells derived from the control iPSCs.

During the neural differentiation A β 1-40 and A β 1-42 levels gradually increased to reach the investigated maximum at day 70 of terminal differentiation (end-point of experiment; TD70) suggesting a maturation-dependent secretion of A β (**Figure 21**). The results at TD14 and TD28 indicated a transition period, where not all AD samples showed significant differences from the controls (**Figure 20**), but from TD42 all AD samples displayed elevated amounts of A β . Therefore, we concentrated our further experimental analyses on the samples matured for a minimum of 6 weeks.

To evaluate the level of APP, the direct precursor of A β in neurons, we performed Western blot analysis. The results revealed that APP expression increased during the neuronal differentiation and reached maximum at day 70 (TD70) in all fAD and sAD neural cultures (**Figure 23**). Expression of this protein was significantly increased in all AD cell lines from TD42. Subsequently, we quantified the amount of APP-CTF generated through APP cleavage by β -secretase. As shown in **Figure 23** remarkably higher APP-CTF protein levels were detected in fAD and sAD neurons. No significant differences in the expression of APP and APP-CTF were observed between fAD and sAD neurons. In summary, both A β and APP levels were significantly elevated in fAD and sAD neural cultures compared to controls. These differences were detectable after 6 weeks (TD42) but were prominent with all sAD lines at week 10 (TD70).

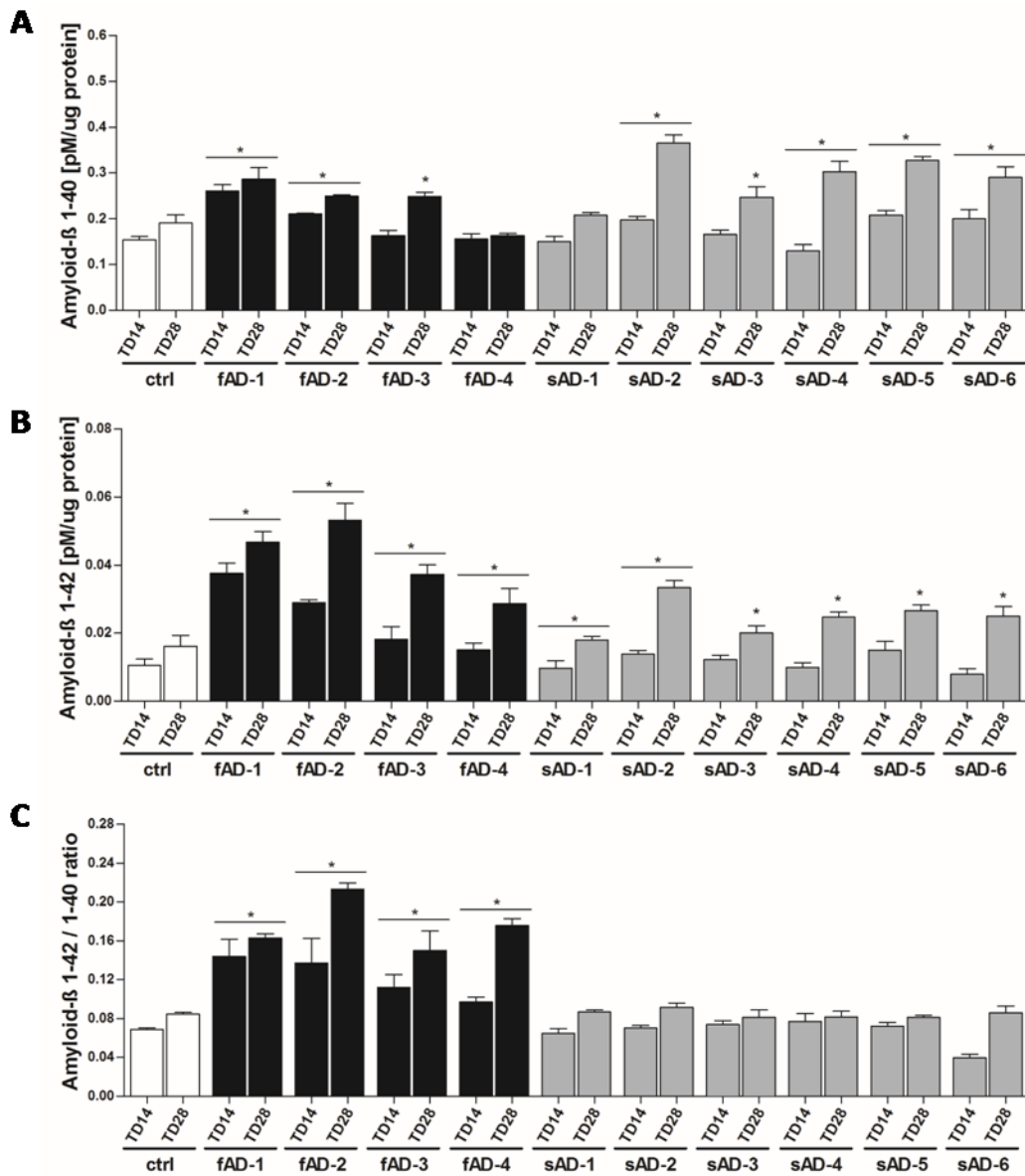


Figure 20. Characterization of A β secretion in control and AD iPSC-derived neurons at TD14 and TD28. (A) The amount of secreted A β 1-40 and (B) A β 1-42 from control- (ctrl-1 – ctrl-4), fAD- (fAD-1 – fAD-4) and sAD- (sAD-1 – sAD-6) -iPSC -derived neurons. (C) The ratio of A β 1-42/A β 1-40 from neurons derived from control and AD lines. A β 1-40 and A β 1-42 secreted from neural cells into the medium were measured at day 4 after the last medium change. The extracellular A β levels determined in pM were normalized to total protein content. Data represent mean \pm S.E.M. (n=3). As the detected level of A β 1-40 and A β 1-42 were not significantly different between the four healthy individual derived clones (ctrl-1, ctrl-2, ctrl-3, ctrl-4), the average value (\pm S.E.M.) as a control value was used in all graphs. Significant differences among groups were examined by Dunnett's test (*p<0.05).

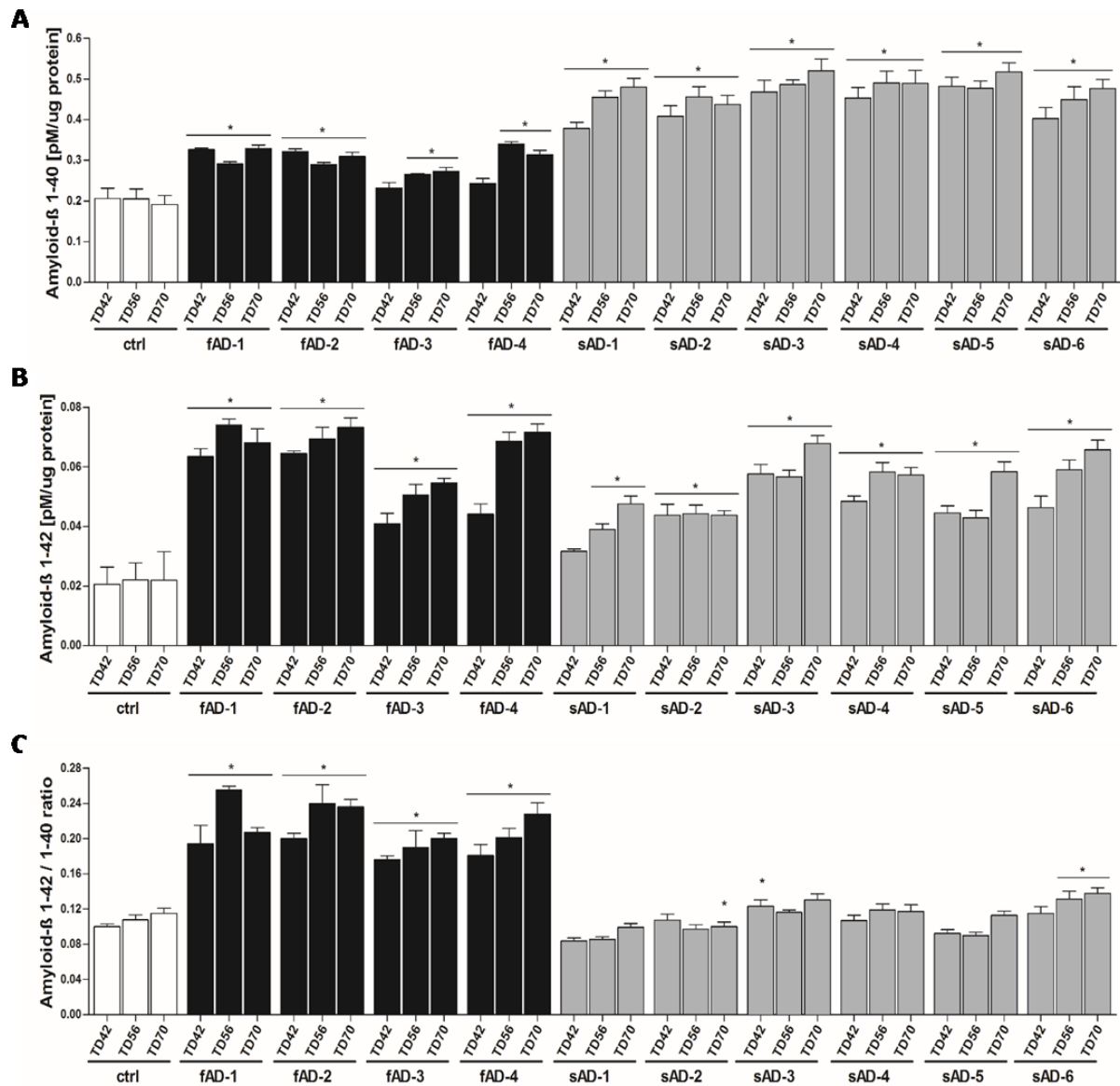


Figure 21. Characterization of A β secretion in control and AD iPSC-derived neurons at TD42, TD56 and TD70. (A) The amount of secreted A β 1-40 from control- (ctrl-1 – ctrl-4), fAD- (fAD-1 – fAD-4) and sAD- (sAD-1 – sAD-6) -iPSC-derived neurons. (B) The amount of A β 1-42 released from control- and AD-iPSC-derived neurons. (C) The ratio of A β 1-42/A β 1-40 from neurons derived from control and AD lines. A β 1-40 and A β 1-42 secreted from neural cells into the medium were measured at day 4 after the last medium change. The extracellular A β levels determined in pM were normalized to total protein content of cell lysates. Data represent mean \pm S.E.M. (n=3). The average value (\pm S.E.M.) from the four healthy individual derived clones (ctrl-1, ctrl-2, ctrl-3, ctrl-4) was used as a control value in all graphs. Significant differences among groups were examined by Dunnett’s test (*p<0.05).

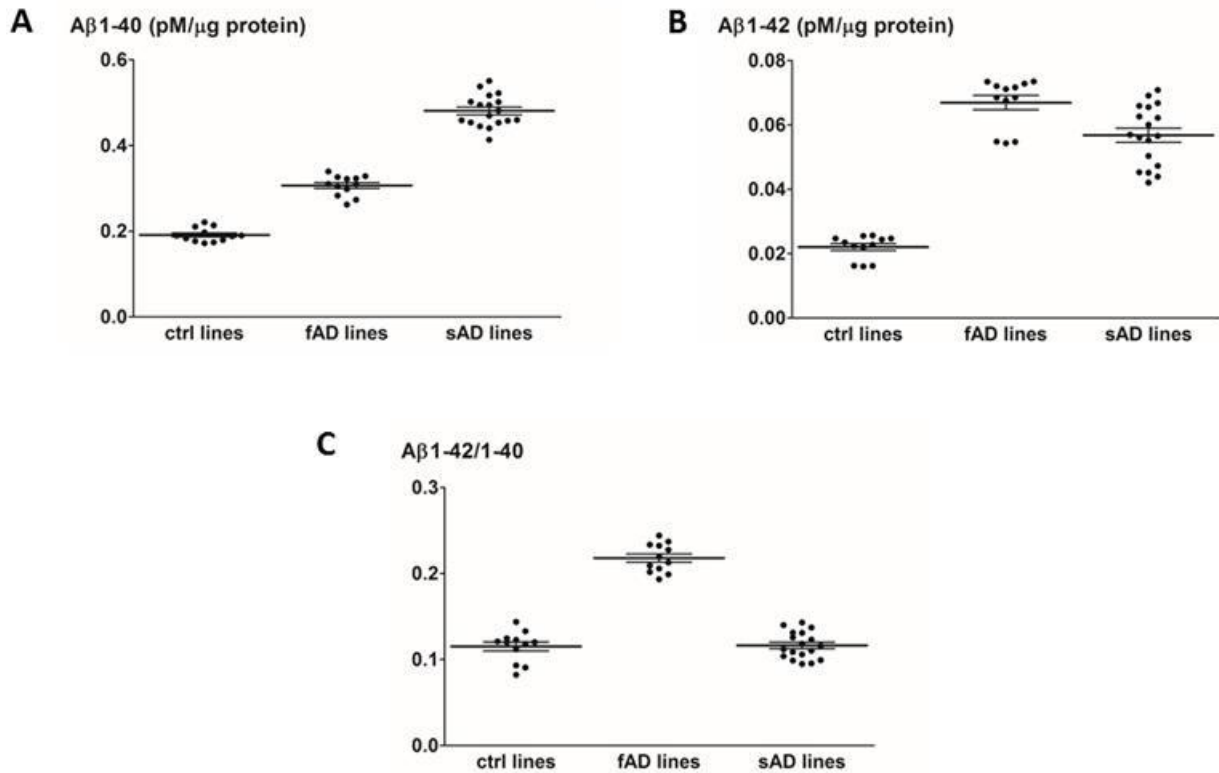


Figure 22. Comparison of the control, fAD and sAD groups of all cell lines studied at day 70 of terminal differentiation (TD70). The individual secretion of A β species in the four non-demented healthy control (ctrl lines), four familiar AD (fAD lines) and six sporadic AD (sAD lines) individuals derived clones. **(A)** The amount of extracellular A β 1-40 and **(B)** extracellular A β 1-42 released from clones into the medium. **(C)** The ratio of A β 1-42/A β 1-40 from neuronal cultures derived from clones. A β 1-40 and A β 1-42 secreted from neural cells into the medium were measured at day 4 after the last medium change. The extracellular A β levels determined in pM were normalized to total protein content. Data represent mean \pm S.E.M. (n=3). Significant differences among groups were examined by Dunnett's test (*p<0.05).

4.2.5 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) as reviewed in (Johnson and Stoothoff, 2004). The appearance and accumulation of abnormally phosphorylated TAU leads to mislocalization and aggregation of TAU, and formation of NFTs (Zempel *et al.*, 2010). Therefore, we focused on the alterations of TAU phosphorylation in our fAD and sAD neurons. TAU protein contains several phosphorylation sites (reviewed in (Sergeant *et al.*, 2005)). Here, we examined six different phosphorylation sites (**Figure 24**) in fAD, sAD and control neurons at different time points of the terminal differentiation. We observed significantly higher phosphorylation of TAU at Ser262 (12E8 epitope) in all our AD neurons compared to controls (**Figure 25A**).

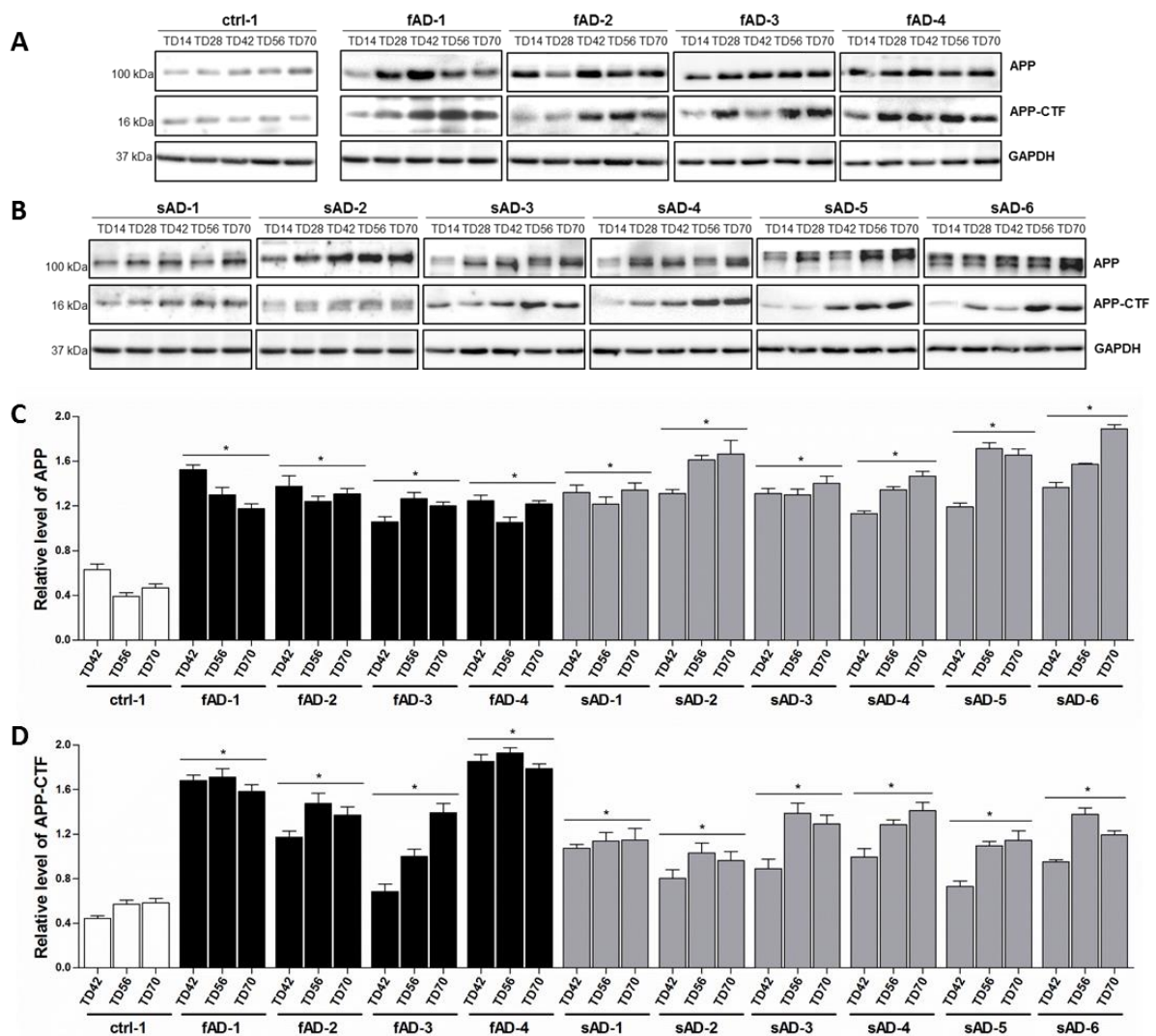


Figure 23. Characterization of APP and APP-CTF expression during neuronal differentiation. (A) Representative immunoblots of APP and APP-CTF in control neurons (ctrl-1), fAD-derived neurons (fAD-1 – fAD-4) and (B) sAD-derived neurons (sAD-1 – sAD-6) at TD14, TD28, TD42, TD56 and TD70. (C) Quantification of APP and APP-CTF signals were normalized to GAPDH. Data are presented as mean \pm S.E.M. ($n = 3$). Dunnett’s method was performed to evaluate the significance of difference between groups (* $p < 0.05$).

Elevated phosphorylation has been detected already at TD42, and reached the highest level between week 8 and 10 (TD56 and TD70) of terminal differentiation. Further analysis revealed upregulated TAU phosphorylation at Ser396 epitope in all familial and sporadic AD neural cells (Figure 25B). Western-blot quantification showed an increase in TAU phosphorylation at Ser202/Thr205 (AT8 epitope) in the AD group (Figure 25C). Furthermore, we observed higher phosphorylation of TAU at Thr181 and Ser400/Thr403/Ser404 in both fAD and sAD-iPSCs-derived neurons (Figure 25D, E). From these results we can conclude that, although the phosphorylation level showed some clonal variations, the pTAU/TAU ratio was significantly

elevated in all AD derived neurons, and no major differences were observed between fAD and sAD iPSC derived neurons.

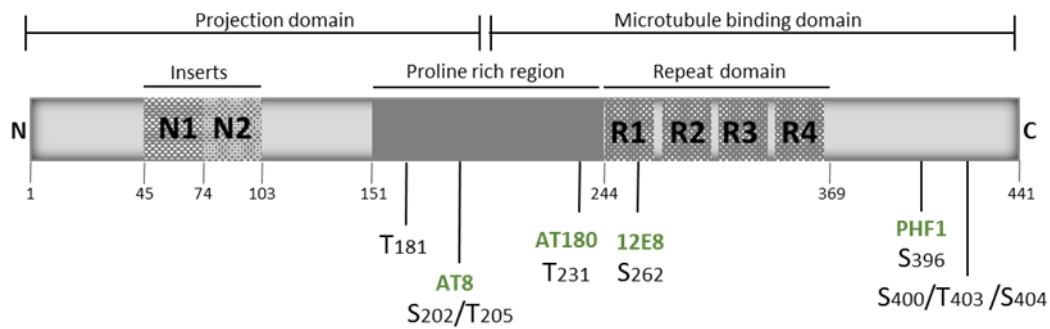


Figure 24. Schematic representation of human TAU isoform (441 amino acids) with the functional projection and microtubule binding domains. Projection domains including a proline rich region and N-terminal part interact with cytoskeletal elements and are involved in signal transduction. Microtubule binding domains with C-terminal part regulate the microtubule polymerization and bind with proteins such as PSEN1. Epitopes of p-TAU antibodies analyzed in this report are indicated on the scheme.

4.2.6 GSK3B activation in AD-derived neurons

Scientific evidences suggest that GSK3B is involved in many pathological hallmarks of AD including hyperphosphorylation of TAU (Rankin *et al.*, 2007), increased A β production (Phiel *et al.*, 2003), memory impairment and neuronal loss as reviewed in (Hooper *et al.*, 2008). To verify if AD neurons with elevated TAU phosphorylation have increased GSK3B activity, the percentage of active form of GSK3B was calculated via assessing the ratio of inhibitory phosphorylation on Ser9 by immunoblotting. Our results demonstrated that neurons derived from patients with fAD and sAD exhibited significantly higher amount of active GSK3B than control neurons as the decreased percentage of inhibitory Ser9 phosphorylation revealed (**Figure 26A, B, C**) in most diseased lines.

During the neuronal differentiation the level of active GSK3B in controls remained constant, while in fAD and sAD cell lines active GSK3B amount has been increasing to reach its maximum at day 56 (sAD-2) or day 70 of the terminal differentiation for all remaining cell lines (**Figure 26C**). Furthermore, higher phosphorylation of TAU at Thr231 (AT180 epitope), which can be catalyzed by GSK3B *in vitro* (Cho and Johnson, 2003) was observed in all fAD and sAD-derived neurons (**Figure 26D**). These results indicate that activation of the GSK3B signaling in both fAD and sAD lines may contribute the abnormal TAU phosphorylation pattern observed in these cell lines.

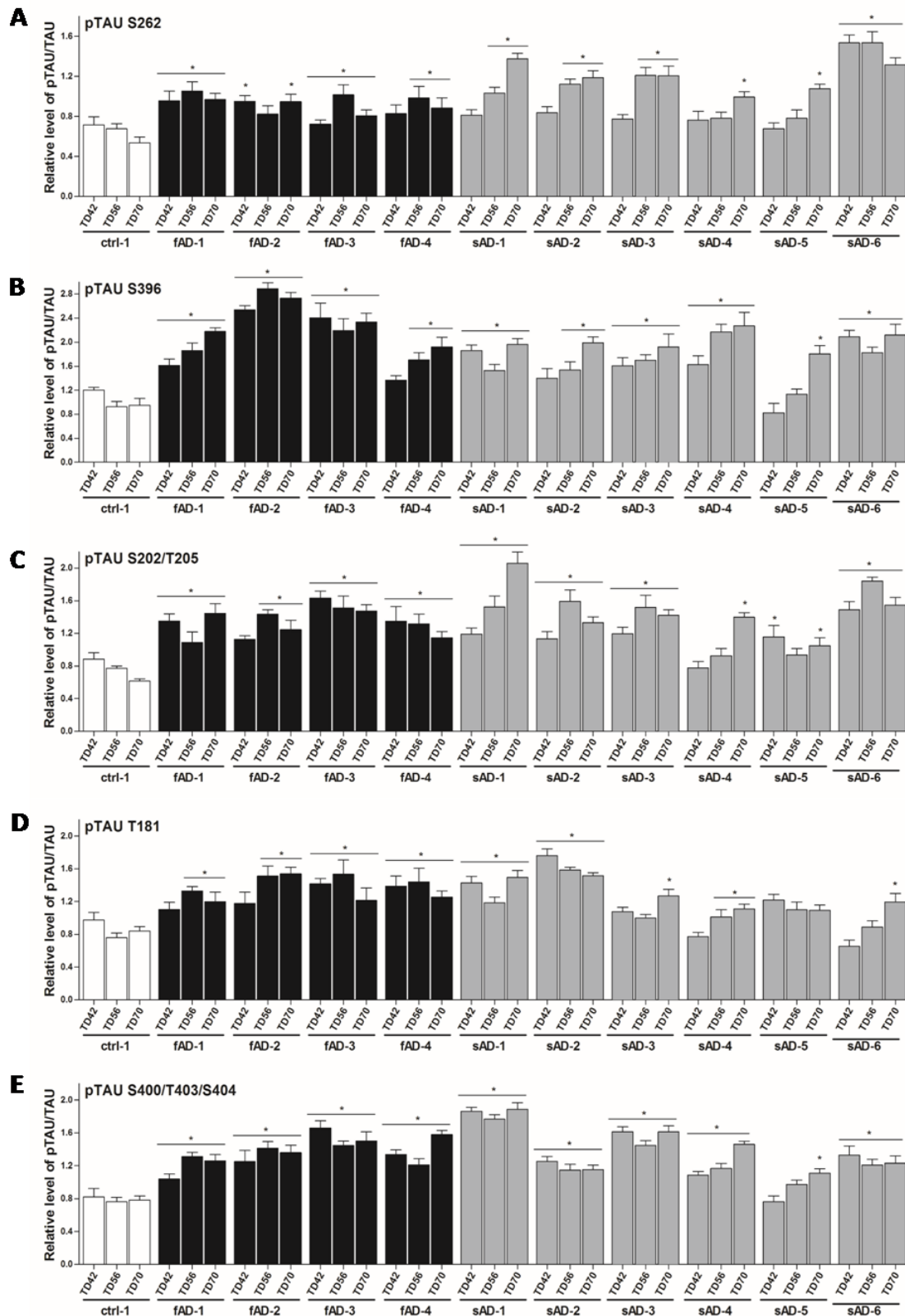


Figure 25. Western blot analysis of total TAU and pTAU protein. (A-E) Densitometry analysis of TAU phosphorylated at different epitopes: S262, S396, S202/T205, T181 and S400/T403/S404 in control (ctrl-1), fAD (fAD-1 – fAD-4) and sAD (sAD-1 – sAD-6) neurons. All samples were analyzed at day 42, 56 and 70 of terminal differentiation. The amount of pTAU relative to total TAU levels in the lysates was measured. GAPDH as the loading control was used to normalize the data. All values are the mean \pm S.E.M. (n=3). Differences of the groups were analyzed with Dunnett's test (* p <0.05).

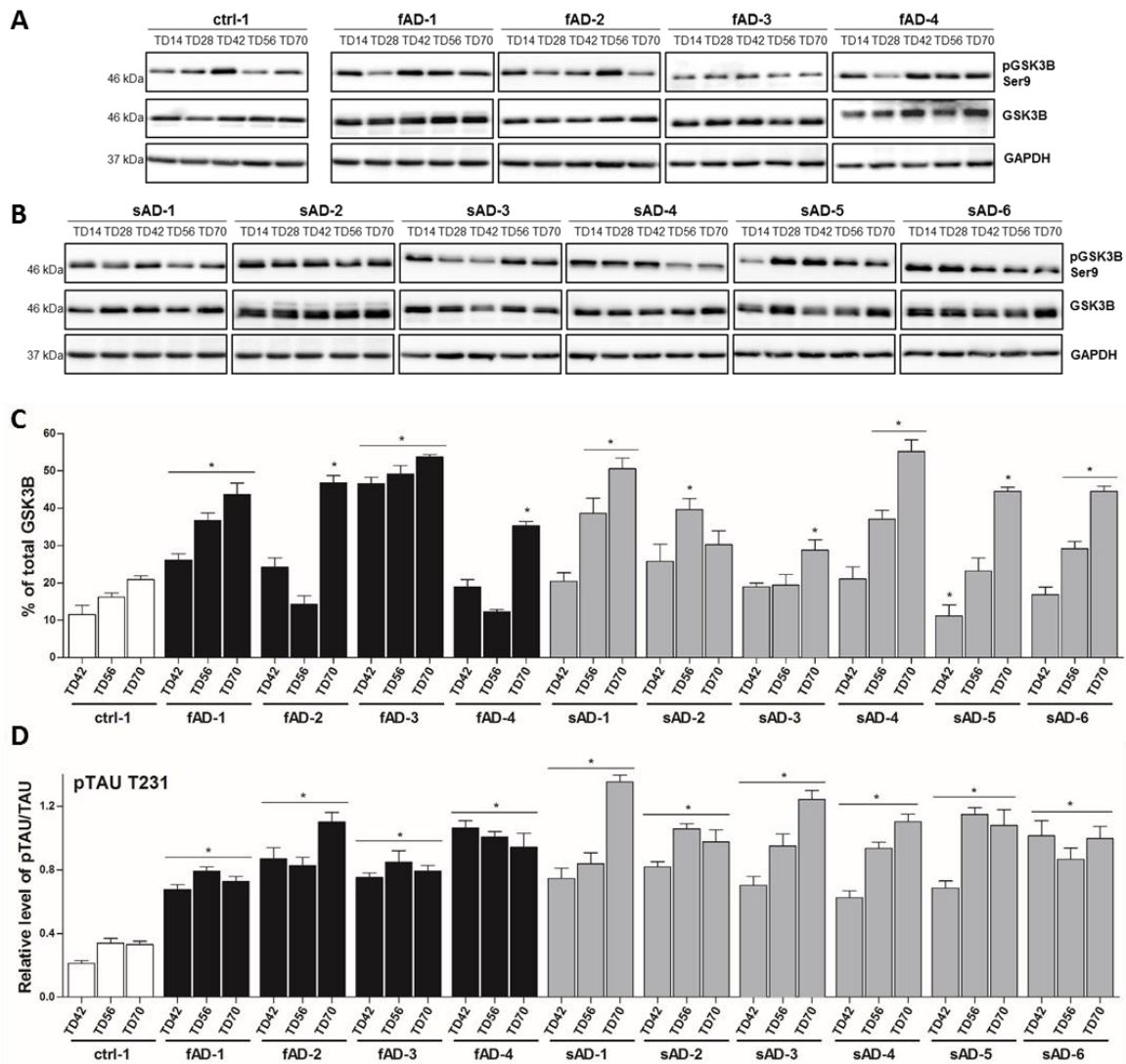


Figure 26. Analysis of GSK3B and active form of GSK3B in neuronal culture. (A) Representative immunoblotting show the phosphorylation of GSK3B at Ser 9 and GSK3B in control neurons (ctrl-1), fAD neurons (fAD-1 – fAD-4) and (B) sAD neurons (sAD-1 – sAD-6) in all time points of neuronal differentiation (TD14 – TD70). (C) Quantification of active GSK3B form at TD42, 56 and 70 was presented as a percentage of non-phosphorylated GSK3B at Ser9. (D) Densitometry analysis of TAU phosphorylated at T231 epitope at day 42, 56 and 70 of terminal differentiation. All values were normalized to GAPDH and are presented as mean \pm S.E.M. ($n = 3$). Significant differences among groups were examined by Dunnett’s method (* $p < 0.05$).

4.2.7 Oxidative stress response in fAD and sAD neuronal cultures

Oxidative stress plays important role in the pathogenesis of neurodegenerative disorders. The molecular mechanism of ROS action in neuronal system has been studied (Fukui *et al.*, 2011; Watson *et al.*, 2012). Despite this, the oxidative stress effect on the human iPSC derived neurons from AD patients is still not well studied. In our experiments we used H_2O_2 in two different concentrations to examine neuronal response to a stressor. In AD and control neurons treatment

with increasing doses of H₂O₂ 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 H₂O₂ compared with controls, cell death caused by 30 μM and 60 μM H₂O₂ reached about 50% and 70% respectively (**Figure 27A, B**).

A series of evidence about the toxicity of Aβ to neurons has been demonstrated in rodent models and neuronal cell lines (Foley *et al.*, 2015; Selkoe and Hardy, 2016). 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 of terminal differentiation for 24 hours with 5 μM Aβ1-42 oligomers showed reduced neuronal survival depending on the neuronal maturation state (**Figure 27C**). Relative to control samples, diseased lines had enhanced sensitivity to cell toxicity induced by Aβ1-42. The 28 days old AD neurons displayed about 15-20% cell death compare to 10% cell loss in control lines. Mature neurons, at TD56, were more susceptible to cell death, almost 40% neurons died in AD cultures, whereas cell loss in control cultures decreased slightly to 15% (**Figure 27C**). Based on our results, we can conclude that fAD and sAD neural cultures are more susceptible to Aβ1-42 oligomer-induced cell death than the controls.

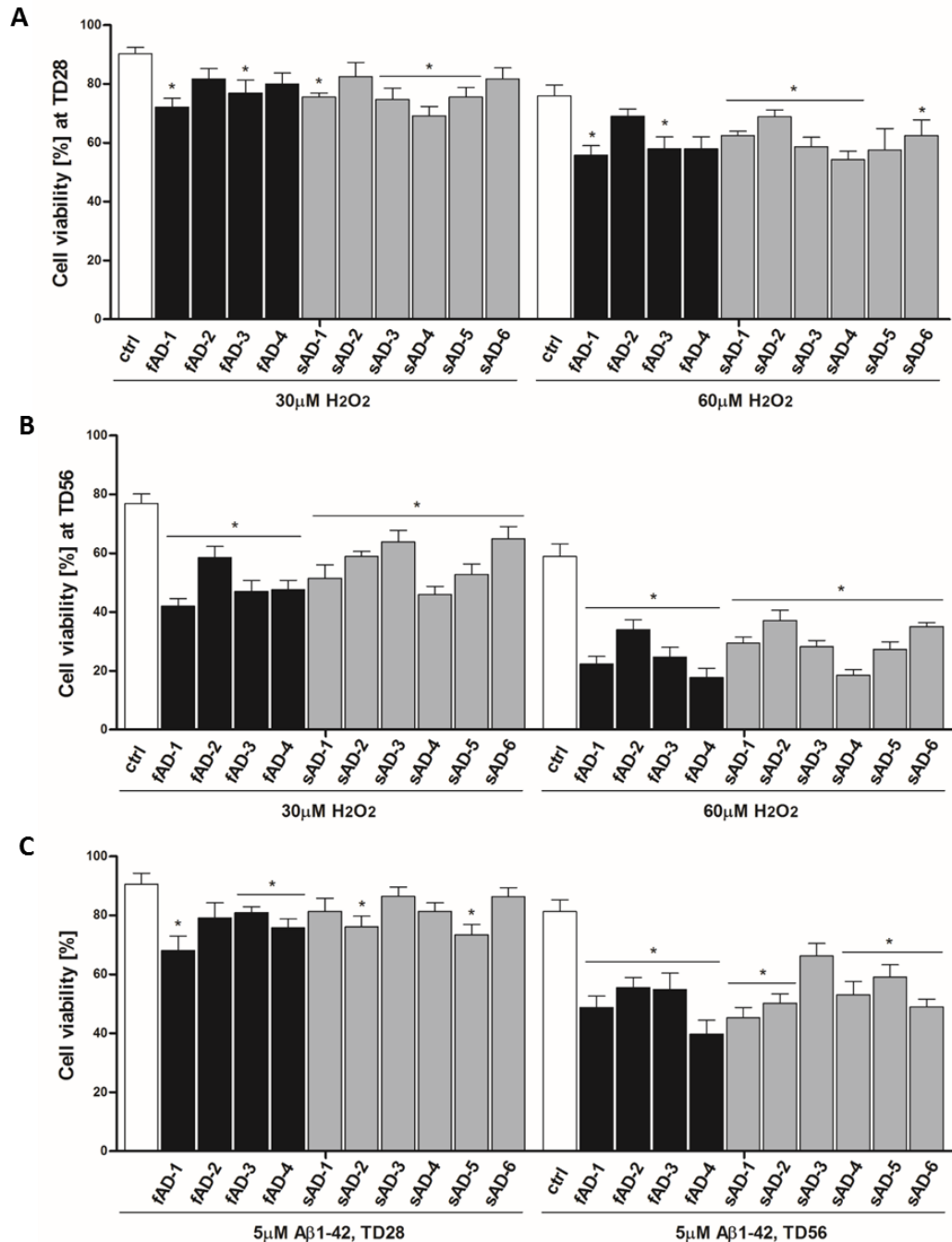


Figure 27. Effect of hydrogen peroxide (H₂O₂) and A β 1-42 oligomer treatment on neuronal viability. (A) Viability of iPSC-derived neurons from controls (ctrl-1 – ctrl-4), fAD (fAD-1 – fAD-4) and sAD (sAD-1 – sAD-6) patients at day 28 (TD28) and (B) 56 of terminal differentiation (TD56) after 24 hours of treatment with 30 μM H₂O₂ and 60 μM H₂O₂. (C) Neuronal survival of control and AD clones at TD28 and TD56 cultured 24 hours in the presence of 5 μM A β 1-42 oligomer solution. The neuronal survival was represented as a percentage of control. Viability of the cultures following H₂O₂ and A β treatment was assessed using a CellTiter-Glo® Luminescent Cell Viability Assay. Values are presented as the mean \pm SD (n = 3). Dunnett’s test was performed to evaluate the significance of groups compared to control (*p<0.05).

5. NEW SCIENTIFIC RESULTS

In this research I studied *in vitro* cellular models of two mammals, the rabbit and human to develop a suitable and efficient disease modelling system. The following new scientific achievements were obtained:

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 A β 1-40 and A β 1-42 secretion was measured in neurons derived from human familiar AD and sporadic AD iPSC lines on a maturation dependent manner. An elevated ratio of A β 1-42 to A β 1-40 was detected in PSEN1 mutants, while A β 1-42/A β 1-40 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 familiar 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.

6. DISCUSSION

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. Over the past decades, primary neurons from animal models and immortalized neuronal cell lines have been used to study neurodegenerative diseases. Due to many limitations, for example genetic and physiological differences between human and rodent brains, the study of AD mechanisms has been controversial. Therefore, there are huge expectations towards iPSC technology, which allows for the generation of pluripotent cells from any individuals in the context of their own genetic identity, to provide new patient specific *in vitro* disease models to study neurological disorders.

Human iPSCs hold great promise for treating neurodegenerative diseases such as Alzheimer's or Parkinson's disease. However, to assess safety, efficacy and long-term effect of transplanted iPSCs, extensive preclinical studies must be applied in animal models before clinical applications. Thus, the rabbit as a laboratory animal model provides a good tool in the study of human disorders.

Rabbits have several advantages compared to other laboratory species. Firstly, they are phylogenetically closer to primates than rodents (Graur *et al.*, 1996) and have been used for many years in biomedical research as experimental models (Weekers *et al.*, 2002; Shiomi *et al.*, 2004). These animals have been held in laboratories for studying human disorders including vascular disease, central nervous system disorders, cardiomyopathy or lipoprotein metabolism dysfunctions (Hoeg *et al.*, 1996; Chen *et al.*, 2001). Secondly, rabbits are larger than rodents that make all physiological manipulations or surgery easier and enable obtain large samples. Furthermore, rabbits have longer life span (7-8 years) than rodents and can be used in long-term treatment in cell based therapies. So far only few groups generated rbiPSCs from somatic cells (Honda *et al.*, 2010; Osteil *et al.*, 2013; Tancos *et al.*, 2017). Besides these advantages, rabbit stem cells are similar to human stem cells in their morphology, biochemical character and molecular mechanisms for self-renewal and pluripotency (Honda *et al.*, 2009).

Recently 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 in forming flattened colonies with sharp edges and growing in media supplemented with bFGF. They also expressed the endogenous pluripotency markers: OCT4, SOX2 and surface protein SSEA4, and were positive for alkaline phosphatase staining. Upon injection into immunodeficient mice they formed teratoma

containing different tissues of all three germ layers. Moreover in the absence of growth factor, the rbiPSCs spontaneously differentiated into ectoderm, mesoderm and endoderm. Taken together, we concluded that rbiPSCs exhibited all cardinal features of pluripotency showing very high similarity to the previously generated and characterized rbiPSCs (Honda *et al.*, 2010; Osteil *et al.*, 2013).

In the next step we investigated if it is possible to generate neurons from rabbit reprogrammed cells. Until now there is no report available about successful production of NPCs or neurons from rbiPSCs. 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 (Kumar *et al.*, 2007; Sathananthan, 2011). 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 such as TUBB3, MAP2 and MUSASHI. 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.

Initially, we attempted to reprogram rabbit somatic cells and provide a new experimental tool for modelling human neurodegenerative diseases. We expected that rabbit iPSCs that closely resemble ESCs and therefore human iPSCs/ESCs can improve understanding of disease mechanism and become a valuable tool in development of therapeutic strategies. However, contrary to our initial concept, we only obtained an ineffective method for the generation of rbiPSC-derived neuronal cells. Without a robust, well-reproducible method we could not investigate the neurodegenerative phenotype. Thus, our effort has been redirected towards human 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 (Yagi *et al.*, 2011; Israel *et al.*, 2012; Kondo *et al.*, 2013; Hossini *et al.*, 2015). In our current study, we analyzed samples and generated neurons from patients carrying the pathogenic mutations in *PSEN1* gene: V89L and L150P (these mutations were first identified by Queralt *et al.* (Queralt *et al.*, 2002) and Wallon *et al.* (Wallon *et al.*, 2012) respectively) and sporadic AD patients with unknown background (**Table 3**). 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 which is in accordance with a previous report (Kondo *et al.*, 2013).

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 A β into extracellular aggregates is one of the pathological sign of AD in the human brain. Alterations in the level of A β peptides are often presented as the ratios between different isoforms. In PSEN mutant mouse model elevated A β production was detected (Duff *et al.*, 1996; Huang *et al.*, 2003; Dewachter *et al.*, 2008). In humans, elevated A β production was revealed in iPSC-derived neuronal lines from familial Alzheimer's disease patients (Yagi *et al.*, 2011), however, only one study demonstrated that A β secretion in sAD-iPSCs-derived neurons is not consistently altered and similar to controls rather than fAD-iPSCs-derived neurons (Kondo *et al.*, 2013). Our study demonstrated an increased extracellular A β 1-40 and A β 1-42 levels in neurons derived from all fAD and sAD lines on a maturation dependent manner. Interestingly, A β 1-40 secretion was approximately 2 fold higher in sAD neurons compared to fAD, while A β 1-42 level remained similar to A β 1-42 level assayed from conditioned media of fAD cell lines. Moreover, we observed an elevated ratio of A β 1-42 to A β 1-40, one of the AD hallmark, in PSEN1 mutants, while A β 1-42/A β 1-40 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. Some groups also detected an increased level of A β 40, A β 42 and A β 42/A β 40 ratio in fAD cell lines with mutations in PSEN1, PSEN2 and APP genes (Yagi *et al.*, 2011; Israel *et al.*, 2012; Kondo *et al.*, 2013). Based on the above results we can conclude that mutations in PSEN1 gene may change the metabolism of A β 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 (Kondo *et al.*, 2013). In fAD, genetic factors modify the clinical phenotype of the disease, while mechanisms underlying the pathogenesis of sAD are still not well understood and combine multiple genetic and environmental risk factors. It is possible that underlying mutations which are not defined yet may play important role in sAD, reflecting the inherent variability of iPSCs. Thus, more cell lines have to be analyzed to reveal the broad heterogeneity of AD 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 (Grundke-Iqbal *et al.*, 1986). TAU phosphorylation at various sites affects TAU activity, its biological function and pathogenic role. Studies on the physiological TAU properties revealed that phosphorylation of Ser262 significantly diminish the ability of TAU to bind microtubules (Biernat *et al.*, 1993), while phosphorylation of few KXGS motifs including Ser262 and Ser356 reduces TAU binding capacity to microtubules and thus increases the dynamics of microtubules, which plays important role in neurite growth and the development of neuronal polarity (Biernat *et al.*, 2002). Phosphorylation at Thr231 leads to decrease the ability of TAU to bind microtubules and reduces the level of acetylated tubulin that consequently leads to microtubule destabilization (Cho and Johnson, 2004). Moreover, the increased TAU phosphorylation at Ser396 and Ser404 impairs microtubule assembly by detachment of TAU molecules from microtubules (Evans *et al.*, 2000). Elevated phosphorylation on Ser262, Thr231 and Ser396 residues could be detectable early in the disease process (Abraha *et al.*, 2000; Augustinack *et al.*, 2002).

A quantitative *in vitro* data revealed a negative impact of TAU phosphorylation at many epitopes on diminishment of TAU activity and microtubule destabilization (Wagner *et al.*, 1996; Drewes *et al.*, 1997; Sengupta *et al.*, 1998). Previous studies on iPSC-derived patient specific fAD and sAD neurons have been limited to detection mostly only one TAU phosphorylation site at Thr231 (Israel *et al.*, 2012; Hossini *et al.*, 2015). 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 (**Figure 23, 24D**): Ser262, Ser202/Thr205, Ser396, Ser400/Thr403/Ser404, Thr181 and Thr231 in all iPSC-derived fAD and sAD neurons. According to the literature, phosphorylation of TAU at Ser262 and Thr231 greatly diminish its ability to bind microtubules by 35% and 25% respectively (Biernat *et al.*, 1993) whereas Ser396 and Ser404 phosphorylation generate more fibrillogenic TAU *in vitro* (Abraha *et al.*, 2000) which shows an increased propensity to aggregate (Haase *et al.*, 2004). Moreover, phosphorylation of AT8 epitope results in decrease of TAU microtubule-nucleation activity leading to microtubule depolymerization and destabilisation (Wada *et al.*, 1998). The increasing TAU phosphorylation during the differentiation, indicates that an appearance of the AD-phenotype depends on the maturation state of neuronal culture. It is in agreement with the *in vivo* studies on mice with mutation in *APP* gene, which displayed accumulation of TAU phosphorylated epitopes within neurites in animals 14 months of age or older (Masliah *et al.*, 2001).

Furthermore, we have shown higher levels of active GSK3B in our AD cultures. This observation is in agreement with previously described findings, which presented increased level of active GSK3B measured in fAD and sAD neurons *in vitro* (Israel *et al.*, 2012) and in transgenic animal models (Ryan and Pimplikar, 2005). Pathological activation of GSK3B establishes a feed-forward loop that contributes to abnormal APP processing (Deng *et al.*, 2014), enhanced apoptosis in hippocampal neurons, TAU hyperphosphorylation and synaptic failure in rodent models of AD (Fuster-Matanzo *et al.*, 2011; Llorens-Martín *et al.*, 2013). Our findings revealed that activation of GSK3B might contribute to TAU misregulation and abnormal phosphorylation. This is in accordance with previous reports confirming the important role of GSK3B in regulating TAU phosphorylation mostly on Thr231 and Ser199, 396, 400, 404 and 413 as reviewed in (Kolarova *et al.*, 2012). Consistent with this, restoring normal level of GSK3B has been shown to reduce TAU hyperphosphorylation, decrease A β production and neuronal death in AD murine models (Engel *et al.*, 2006) as well as decrease A β -induced neurotoxicity in cultured mouse primary neurons *in vitro* (Koh *et al.*, 2008).

In our study we evaluated cell viability after hydrogen peroxide and extracellular A β 1-42 exposure in neurons at different maturation stages. Exposure to stress agents such as H₂O₂ induces ROS production and toxicity in many different cell types (Lee *et al.*, 2001; Kim *et al.*, 2003; Tochigi *et al.*, 2013). ROS show high reactivity with macromolecules and play important role as signaling molecules as reviewed in (Uttara *et al.*, 2009). Oxidative damage is linked with mitochondrial abnormalities and is catalyzed by the presence of metal ions Fe and Cu. Our results demonstrated a significant H₂O₂ dose-related decrease in the survival of fAD and sAD

neurons. More mature neurons (TD56) showed a greater sensitivity to H₂O₂ than younger neuronal cultures (TD28). These observations indicate that H₂O₂ 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 A β 1-42 oligomers induced cell death both in fAD and sAD neurons. However, a decreased cell survival was detected in mature (TD56) neurons. According to the literature, A β treatment may lead to activation of glutamate receptors and inhibition of glutamate transporters that leads to abnormal release of glutamate and disturbances in glutamatergic neurotransmission. A β treatment induces NMDA-receptor mediated cellular events in neurons and astrocytes leading to synaptic damage and spine loss (Talanta *et al.*, 2013). Chronic stimulation of NMDA receptor results in Ca²⁺ influx which activates apoptotic pathways and increased glutamate excitotoxicity leads to generation of ROS leading to neuronal damage and cell death (Mailly *et al.*, 1999). In cortical neurons, accumulation of glutamate and NMDA receptors promotes H₂O₂ mediated neurotoxicity and oxidative damaged in DNA.

Furthermore, increased influx of Ca²⁺ mediated by A β treatment activates mitochondrial permeability transition pore (PTP) leading to deregulation of respiratory chain enzymes and ROS overproduction, and consequently neurotoxicity (Morkuniene *et al.*, 2015). Based on the above data we can speculate, that the neuronal death observed in our cultures upon the synthetic A β treatment may be a consequence of mitochondrial stress and higher ROS production.

It has previously been reported that accumulated A β oligomers induce endoplasmic-reticulum (ER) stress and ROS production (Nishitsuji *et al.*, 2009), which leads to membrane lipid peroxidation and impairment of membrane protein function as reviewed in (Butterfield, 2003). Furthermore, gene analysis of the APP E693 Δ neurons revealed upregulation of the oxidative stress-related markers (Kondo *et al.*, 2013). Additionally, *in vivo* studies reported increased protein oxidation and lipid peroxidation in PSEN1 mutant brain (Mohmmad *et al.*, 2004; Schuessel *et al.*, 2006) leading to destruction of spine morphology and impaired synaptic plasticity (Auffret *et al.*, 2009). Thus, we speculate, that our fAD and sAD-derived neurons can also exhibit increased level of the stress-related genes suggesting ER and Golgi perturbation. It is worth considering that mutation in PSEN1 and pathological changes in sporadic AD combined with neuronal aging can upregulate ROS production leading to mitochondrial damage which may contribute to the neurodegenerative processes and AD progress.

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 A β 1-40 and A β 1-42 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.

7. SUMMARY (EN)

The aim of the current study was to establish rabbit and human neuron based *in vitro* cellular models to study the pathomechanism of neurodegenerative diseases. The most common disorder behind dementia in humans, the Alzheimer's disease was chosen, which affects one in eight adults over 65. 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. First, rabbit embryonic fibroblasts were reprogrammed by lentiviral transduction with the OSKM human transcription factors. Newly generated rbiPSC line exhibited typical pluripotent stem cell characteristic both *in vitro* and *in vivo* in teratoma assay. Induced differentiation of rbiPSCs into neuronal lineage, investigated with several methods eg. suspension culture, hanging drop method or dual SMAD inhibition in monolayer culture has shown low efficiency of neuronal induction. The highest number of neuronal rosettes was achieved using SMAD inhibition protocol with small molecules, as a few cells expressed the neuronal markers. Due to the inefficient differentiation capacity and hard reproducibility of the neuronal differentiation, hiPSC were used for further studies. Human iPSCs generated from blood samples of AD patients and control individuals were successfully differentiated towards mature cortical neurons. In our knowledge this is the first analysis which revealed that neurons derived from familial AD (fAD) and sporadic AD (sAD) patients showed increased phosphorylation of TAU protein in all investigated phosphorylation sites. Compared to control lines, fAD and sAD neurons exhibited higher levels of extracellular amyloid β 1-40 (A β 1-40) and amyloid β 1-42 (A β 1-42), and significantly increased A β 1-42/A β 1-40 ratio that is one of the pathological markers of the disease. Additionally, increased levels of active glycogen synthase kinase 3 beta (GSK3B), a physiological kinase of TAU in neurons derived from AD-iPSCs, and a significant upregulation of amyloid precursor protein (APP) synthesis and APP carboxy-terminal fragment (APP-CTF) cleavage was detected. Moreover, elevated sensitivity to oxidative stress, induced by amyloid oligomers or oxygen peroxide was shown both in familial and sporadic AD-iPSCs differentiated neurons. The results of this study provide strong evidence that iPSC technology can be used in modelling of both fAD and sAD cases. In Hungary this is the first study on AD which generated and studied human iPSCs from familial and sporadic AD patients. This new strategy will allow to study pathological mechanism on the molecular level and analyze patient derived neurons *in vitro*. The iPSCs have great potential for improving the model systems used in preclinical and clinical stages of neurodegenerative disease drug development.

8. SUMMARY (HU)

Az értekezés célja egy olyan nyúl és humán neuron alapú *in vitro* sejtes modellrendszer létrehozása volt, amely lehetővé teszi a neurodegeneratív betegségek pathomechanizmusának modellezését. Ennek érdekében az egyik leggyakoribb demencia, az Alzheimer kór (AD) modellezését választottuk, amely minden nyolcadik 65 év feletti embert érint. Az AD betegek zöme ún. sporadikus eset, ismeretlen genetikai háttérrel, az AD monogénes, ún. familiális változata csak az esetek 5%-át teszi ki. Ezért egy megfelelő fenotípusos modell szükséges ahhoz, hogy a betegség pathomechanizmusa vizsgálható legyen. Kutatásunk során először nyúl májsejtekből humán OSKM faktorokat felhasználva, lentivirális újraprogramozással, iPSC sejteket állítottunk elő, amelyek mind *in vitro*, mind *in vivo* vizsgálatokban pluripotensnek bizonyultak. A nyúl iPSC sejtek neurális differenciációját több módszerrel is indukáltuk, például szuszpenziós, függőcseppes, vagy duális SMAD inhibícióval egysejtrétegű kultúrákon. A legmagasabb neurális rozetta számot a SMAD inhibíciós protokoll eredményezte kismolekulák alkalmazásával, így néhány sejt expresszáta a neurális markereket. . Így az alacsony hatékonyságú és nehezen reprodukálható nyúl rendszer helyett humán iPSC sejtekkel folytattuk a kísérleteket. Alzheimer betegek és kontroll személyek vérmintáinak újraprogramozása révén előállított iPSC sejt vonalakat kortikális neuronokká differenciáltattunk. Legjobb tudomásunk szerint ez az első olyan kísérletsorozat, ahol megnövekedett TAU fehérje foszforilációt sikerült kimutatni több TAU epitópon, mind a sporadikus és familiális AD betegek neuronjain. Ezen felül a betegség másik jellemzője, az extracelluláris A β 1-40 és A β 1-42 megnövekedett mennyiségét detektáltuk, az A β 1-42/A β 1-40 arány változásával egyetemben. E mellett az AD betegekben megnövekedett aktív GSK3B szintet (amely a TAU fehérje fiziológias kináza), APP szintet és APP-CTF szintet detektáltunk. További vizsgálataink megmutatták, hogy mind a sporadikus, mind pedig a familiális AD neuronok fokozottan érzékenyek az oxidatív stresszre, akár oligomeramiloid, akár hidrogén-peroxid indukálta azt. Eredményeink bizonyítják, hogy az iPSC technológia nemcsak a monogénes familiális, de az ismeretlen genetikai hátterű sporadikus AD modellezésére is alkalmas módszer. Magyarországon ez az első kutatás, amely iPSC sejteket használ fel az AD kutatásában. Ez az új stratégia lehetőséget nyújt arra, hogy egy betegség patológiájának molekuláris folyamatait tanulmányozzuk humán neuronokon. Az iPSC sejteknek nagy jelentősége van a betegségmodellek tökéletesítésében, a pre-klinikai és klinikai neurodegeneratív betegség gyógyszer fejlesztések területén.

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10. 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, Kobolák J, Dinnyés 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, Dinnyés 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, Kobolák J, Dinnyés 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, Kobolák 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, Berzsényi 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
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- 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
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11. APPENDICES

Appendix A:

Supplementary Table 1. Antibodies used for immunocytochemistry and immunoblotting.

	Antibody	Dilution	Company (Cat#)
Immunocytochemistry (ICC)	Mouse anti-SOX2	1:200	Santa Cruz (SC-365823)
	Mouse anti-OCT4	1:50	Santa Cruz (SC-5279)
	Mouse anti-SSEA1	1:100	Abcam (AB16285-200)
	Mouse anti-SSEA4	1:1000	Hybridoma Bank (MC-813-70)
	Rabbit anti-MUSASHI	1:1000	Merck Millipore (AB5977)
	Rabbit anti-SMA	1:100	Abcam (AB5694)
	Mouse anti-GATA4	1:50	Santa Cruz (SC-25310)
	Goat anti-GFAP	1:50	Santa Cruz (SC-6170)
	Mouse anti-NESTIN	1:1000	Merck Millipore (MAB5326)
	Rabbit anti-PAX6	1:500	Covance (PRB278P)
	Rabbit anti-MAP2	1:1000	Merck Millipore (MAB3418)
	Rabbit anti- β -III-TUBULIN	1:1000	Covance (PRB-435P)
	Mouse anti- β -III-TUBULIN	1:1000	Santa Cruz (SC-58888)
	Rabbit anti-TAU	1:1000	Dako (A002401)
	Rabbit anti-NF200	1:1500	Abcam (AB8135)
	Rabbit anti-GABA	1:1000	Abcam (AB43865)
	Rabbit anti-GAD 65/67	1:1000	Abcam (AB49832)
Rabbit anti-VGLUT 1/2	1:500	Synaptic Systems (135503)	
Rabbit anti-VACHT	1:1000	Synaptic Systems (139103)	
Immunoblotting (WB)	Mouse anti-Tau-5	1:1000	Calbiochem (577801)
	Rabbit anti-pTau S262	1:500	Merck Millipore (AB9656)
	Mouse anti-pTau S202/T205	1:500	Thermo Fisher (MN1020)
	Rabbit anti-pTau S400/T403/S404	1:1000	Cell Signaling (11837S)
	Rabbit anti-pTau S396	1:15000	Abcam (AB109390)
	Rabbit anti-pTau T181	1:1000	Thermo Fisher (PA1-14413)
	Mouse anti-pTau T231	1:250	Thermo Fisher (MN1040)
	Mouse anti-GSK-3 β	1:1000	Cell Signaling (9832)
	Rabbit anti-pGSK-3 β (S9)	1:1000	Cell Signaling (9323)
	Mouse anti-APP/ β -amyloid	1:1000	Cell Signaling (2450)
Rabbit anti-APP-CTF	1:1000	Thermo Fisher (51-2700)	

Supplementary Table 2. Gene specific human primers used for qRT-PCR analysis.

Gene ID	Primer name	Forward sequence	Reverse sequence
2597	GAPDH	5'-CTCTCTGCTCCTCCTGTTTCGAC-3'	5'-TGAGCGATGTGGCTCGGCT-3'
10763	NESTIN	5'-ACTGAAGTCTGCGGGACAAG-3'	5'-CAGTGGTGCTTGAGTTTCTG-3'
10381	TUBB3	5'-AACGAGGCCTCTTCTCACAA-3'	5'-GGCCTGAAGAGATGTCCAAA-3'
4133	MAP2	5'-TTGTCTCTAACCGAGGAAGCA-3'	5'-TCGTTGTGTCGTGTTCTCAA-3'
2902	GRIN1	5'-GCAACACCAACATCTGGAAG-3'	5'-ATCCGCATACTTGGAAGACA-3'
146713	RBFOX3	5'-GGAGAAGCTGAATGGGACGA-3'	5'-GCCGTGGCATTATGACCT-3'
1742	DLG4	5'-ACAAGCGGATCACAGAGGAG-3'	5'-AGTCTCTCTCGGGCTGGAAC-3'
6532	SLC6A4	5'-GTGAGGATGTGGATGGAGGT-3'	5'-GCGAGATAGCATCCCTGTTC-3'
2571	GAD1	5'-GCACAGGTCATCCTCGATTT-3'	5'-TTGATGTCAGCCATTCTCCA-3'
7054	TH	5'-TCCACGCTGTACTGGTTCAC-3'	5'-GCACCATAGGCCTCACCTC-3'
1103	CHAT	5'-GCCTGCTGCAATCAGTTCTT-3'	5'-GTCCTCGTTGGAAGCCATT-3'
2670	GFAP	5'-CGTCTGGATCTGGAGAGGAA-3'	5'-ACCTCCTCCTCGTGGATCTT-3'
5010	CLDN11	5'-TTCTGAAATCCTCAATTCATC-3'	5'-ACATCATACAAACCTGAAATCAA-3'

Appendix B: Media compositions

1. Thawing Medium (TM)

Reagent	Concentration	For 10 ml
Complete NMM media	100 %	10 ml
Rock inhibitor (10mM)	end concentration 10 μ M	

2. Freezing Medium (FM)

Reagent	Concentration	For 10 ml
FBS	90 %	9 ml
DMSO	10 %	1 ml
Rock inhibitor (10mM)	end concentration 10 μ M	

3. Neural Induction Medium (NIM)

Reagent	Concentration	For 500 ml
DMEM/F12	50 %	235 ml
Neurobasal medium	50 %	235 ml
Nonessential amino acids	1 %	5 ml
B-27 (50x)	2 %	10 ml
N-2 (100x)	1 %	5 ml
L-Glutamine (200mM)	1 %	5 ml
Pen/Strep	1 %	5 ml
SB431542 10mM	end concentration 10 μ M	
NOGGIN 100ug/ml	end concentration 500ng/mL	
LDN193189 100ug/ml	end concentration 10ng/mL	
β -mercaptoethanol	end concentration 100 μ M	
Insulin	end concentration 5 μ g/ml	

4. Neural Maintenance Medium (NMM)

Reagent	Concentration	For 500 mL
DMEM/F12	50 %	235 mL
Neurobasal medium	50 %	235 mL
Nonessential amino acids	1 %	5 ml
B-27 (50x)	2 %	10 ml
N-2 (100x)	1 %	5 ml
L-Glutamine (200mM)	1 %	5 ml
Pen/Strep	1 %	5 ml
EGF 100ug/ml	end concentration 10ng/mL	
bFGF 100ug/ml	end concentration 10ng/mL	
β -mercaptoethanol	end concentration 100 μ M	

5. Neuronal Differentiation Medium (NDM)

Reagent	Concentration	For 500 ml
DMEM/F12	50 %	235 ml
Neurobasal-A medium	50 %	235 ml
Nonessential amino acids	1 %	5 ml
B-27 (50x)	2 %	10 ml
N-2 (100x)	1 %	5 ml
L-Glutamine (200mM)	1 %	5 ml
Pen/Strep	1 %	5 ml
Ascorbic Acid	end concentration 0.2mM	
β -mercaptoethanol	end concentration 25 μ M	

Appendix C: Ethics

The human iPSC lines used in this study were generated in the research laboratory of BioTalentum Ltd.

BioTalentum Ltd. has generated patient-specific iPSC lines with patient consent for research and industrial use. The ethical permissions are valid and has been issued by the Hungarian National Health Scientific Council (ETT-TUKEB 834/PI/09, 8-333/2009-1018EKU; and 314/2014 (31203/1/2014/EKU). The cell lines were fully anonymized and handled according to the ethical and legal requirements.

Here, in the current study we used the samples of clinically characterized Alzheimer's disease patients, diagnosed and sampled by the Institute of Genomic Medicine and Rare Disorders, Semmelweis University, Budapest (Hungary).

12. ACKNOWLEDGEMENTS

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 incited 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 Teglassi 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.

This work was supported by grants from EU FP7 projects (STEMMAD, PIAPP-GA-2012-324451; EpiHealth, HEALTH-2012-F2-278418; EpiHealthNet, PITN-GA-2012-317146) and Szent Istvan University (Research Center of Excellence 11476-3/2016/FEKUT) projects.