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NEURAL DIFFERENTIATION OF MOUSE AND HUMAN PLURIPOTENT STEM CELLS

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ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
Aβ	Amyloid-beta
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMD	Age-related macular degeneration
A–P	Anterior–posterior
APP	Amyloid precursor protein
ASD	Autism spectrum disorder
AQP4	Aquaporin 4
BDNF	Brain-Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
CNS	Central nervous system
d	Day
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
D-V	Dorso-ventral
EB	Embryoid body
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
ESC	Embryonic Stem Cell
FACS	Fluorescence-activated cell sorting
fAD	Familial Alzheimer's disease
FBS	Foetal Bovine Serum
FCS	Fetal calf serum
FGF	Fibroblast Growth Factor
GFAP	Glial Fibrillary Acidic Protein
GLAST	Astrocyte-specific Glutamate Transporter
hESC	Human embryonic stem cells
HMG	High mobility group
ICC	Immunocytochemistry
ICM	Inner cell mass
INs	Interneurons
IP	Intermediate progenitor
iPSCs	Induced pluripotent stem cells

LGIC	Ligand-gated ion channel
LIF	Leukemia Inhibitory Factor
MAP	Microtubule-associated Protein
MEF	Mouse embryonic fibroblasts
NCC	Neural crest cell
Neo	Neomycin
NEP	Neuroepithelial progenitors
NIM	Neural induction media
NMM	Neural maintenance media
NPCs	Neuronal progenitor cells
OSKM	OCT4/SOX2/KLF-4/c-MYC
PBS	Phosphate-buffered saline
PBMC	Peripheral blood mononuclear cell
PC	Pyramidal cell layer
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDGF	Platelet-derived Growth Factor
PFA	Paraformaldehyde
POL	Poly-ornithine
POL/L	Poly-L-ornithine and laminin
PSCs	Pluripotent stem cells
PSEN1	Presenilin-1
PSEN2	Presenilin-2
RA	Retinoic acid
RG	Radial glial cells
RNA	Ribonucleic acid
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RT-PCR	Reverse transcription polymerase chain reaction
RT	Room temperature
sAD	Sporadic Alzheimer’s disease
SCF	Stem Cell Factor
SCID	Severe combined immunodeficiency
SCNT	Somatic cell nuclear transfer
SPs	Senile plaques
TE	Trophectoderm
TGF-β	Transforming growth factor-β

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1. INTRODUCTION AND OBJECTIVE

1.1 Importance of the field

For more than decades, nearly every medical breakthrough in human and animal health has been the direct result of research using animals. The use of animal models in research allows the researcher to investigate the state-of-the-disease in ways, which are inaccessible to humans. Although the use of animals as models for human anatomy and physiology began in ancient Greece, it intensified only by the beginning of the twentieth century. Since then, the use of animal modeling had dramatically increased, particularly in rodents (mouse and rat) where they had become the fashionable method of demonstrating biological significance. Scientists across the biomedical fields are using the mouse model due to its close genetic and physiological similarities to humans, as well as the ease with which its genome can be manipulated and analyzed in a controlled environment. Until now there have been various animal models used in analyzing human disease, such as *Drosophila melanogaster* and *Caenorhabditis elegans* invertebrate model organisms for human genetics (Pandey and Nichols, 2011), *Danio rerio* as a vertebrate model for drug assessments, and for gene functions (Santoriello and Zon, 2012). However, two families of mammals, the rodents (mouse and rat) (Chesselet and Carmichael, 2012) and *Leporidae* (Rabbit and Hare) (Carneiro *et al.,* 2011) are the most frequently used human animal models.

Although animal experiments remain vital in biomedical research, there is a general agreement that use of animals must be restricted to the necessary minimum. In 1959, William Russell and Rex Burch published the idea of the "3Rs"- Reduction, Refinement, and Replacement (William and Burch, 1959). They proposed that if animals has to be used in experiments, every exertion should be made to "*Replace*" them with alternatives such as computer modeling, *in vitro* methodologies; "*Reduce*" to minimize the number of animals used per experiment e.g. by data and resource sharing and "*Refinement*" altering how animals are used in the experiments, as they should be exposed to minimal pain e.g. use of non-invasive techniques. The animal welfare act for the Replacement, Refinement, and Reduction of Animals in Research helps co-ordinate best practice on the 3Rs throughout Europe and UK.

Though small-animal models, like laboratory mouse and rat, offer apparent advantages regarding high reproductive rates, low maintenance costs, and the ability to perform experiments using inbred genetically identical animals, the species specific differences can cause relevant differences from humans (Mestas and Hughes, 2004). Especially extrapolating these results to human disease is often not straightforward. Some of these limitations have been overcome by the advances in the development of transgenic mice that have been reconstituted with the human immune system (Strowig *et al.,* 2010; Schulz *et al.,* 2012). However, the clinical translation of rodent data are still problematic, sometimes causing major failures in drug development. The demand for alternative methods for animal experiments has become increasingly strident in recent decades. As an alternative approach, "*in vitro* cell culture" can provide a reliable mechanistic insight of the disease without culling the animal. The *in vitro* cell culture is a technique where cells or tissue is fragmented from the living organism and is cultured in an artificial environment. The cultured tissue may consist of either single cells, a population of cells, or a whole part of an organ (Henle and Deinhardt, 1957; Ranganatha and Kuppast, 2012; Doke and Dhawale, 2015). This technique offers an excellent model system for studying

metabolic processes, aging, mutagenesis, and carcinogenesis (Kirsch-Volders *et al.,* 2011; Sant'Anna *et al.,* 2015).

1.2 The development of pluripotent *in vitro* cell culture models

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of an embryo at the blastocyst stage (M. J. Evans and Kaufman, 1981; Thomson *et al.*, 1995). They are characterized by the ability to proliferate indefinitely (or self-renew themselves) *in vitro* while maintaining pluripotency (Thomson *et al.*, 1998; Hall, 2016). In parallel, they have the ability to differentiate into three germ layers through asymmetric cell divisions, the endoderm, mesoderm, and ectoderm and can classify into any cell type both *in vivo* and *in vitro* (Siller *et al.*, 2013; Hall, 2016).

Reprogramming adult human somatic cells to induced pluripotent stem cells (iPSCs) is a novel approach to produce patient-specific pluripotent cells, which might be suitable for autologous transplantation. Induced pluripotent stem cells can be generated from lineage-restricted cells through the ectopic expression of defined transcriptional factors. The goal of regenerative medicine is to regenerate fully function a tissues or organ that can replace the lost or damaged ones during diseases, injury or aging (Dowey *et al.,* 2012). The enthusiasm for producing patient-specific human embryonic stem cells using somatic nuclear transfer has somewhat abated in recent years because of ethical, technical, and political concerns. However, the interest in generating iPSCs, in which pluripotency can be obtained by transcription factor overexpression of various somatic cells, has rapidly increased. Human iPSCs are anticipated to open enormous opportunities in the biomedical sciences regarding cell therapies for regenerative medicine and stem cell modeling of human disease (Huangfu *et al.*, 2008; Fusaki *et al.*, 2009).

Although several methods are published to differentiate pluripotent cells into different cell types, both in mouse and human, the cell line specific differentiation protocols are not well established. It means though the promise of clinical use is reliable the exact methodology is still not available. Currently our understanding of cell differentiation decisions, which drive the cells towards specific lineages, as well as the decisions and pathways behind the pluripotency, are not well understood. Further comprehensive studies require developing new strategies and tools. Therefore, standardized methods must be developed to characterize pluripotent stem cells (PSCs) and their derivatives. Furthermore, cellular reprogramming (generation of iPSCs) has demonstrated a proof-of-principle, but the process is not standardized yet to transition into clinical trials. Thus, unraveling the molecular mechanisms that govern reprogramming is a critical first step toward standardizing protocols.

The development of *in vitro* human models hinges on the availability of tissue and organ-specific cell types that could provide insights into disease phenotypes and mechanism for treatments. To date, most of the tissue engineering strategies rely on primary cells derived from diseased vs. healthy patients (Hossini *et al.*, 2015). Primary cells are the most physiologically relevant to the tissue, however, are difficult to obtain, proliferate, and often have limited life span (Yuan *et al.*, 2013). Additionally, in many cases, biopsies represent the end stage of the disease and control tissue is obviously inaccessible due to ethical concerns and potential health risks (Hossini *et al.*, 2015). Given such practical limitations, human PSCs can provide significant opportunities to overcome these limitations (Takahashi and Yamanaka, 2006). In particular, human embryonic stem cells (hESC) have considerable potential for transplantation therapies (Sundberg *et al.*,

2009; Lappalainen *et al.*, 2010), although hiPSC-based therapies are developing quickly (Hata *et al.*, 2017; Mandai *et al.*, 2017; Wakazono *et al.*, 2017). Both types of human pluripotent stem cells (e.g., hESCs and hiPSCs) are not only possible cell sources for transplantation therapies but also have a great potential as *in vitro* toxicity and drug testing models and for use in developmental studies, disease modeling, and patient-specific diagnostics (Bal-Price *et al.*, 2010; Johnstone *et al.*, 2011; Zagoura *et al.*, 2016).

1.3 Setting up an efficient neuronal lineage-specific culture system

Cellular-based assays have been an important milestone in the disease modeling process to provide a simple and cost-efficient tool to avoid cost-intensive animal testing. To date, the majority of cell-based assays use the classical two-dimensional (2D) monolayer cultures on flat and rigid substrates, which allows cells to interact in only two directions, thereby resulting in fewer connections between cells. In the case of neuronal 2D cultures, this result in longer neuronal processes increased proliferation and decreased maturation compared to those in three-dimensional cultures (3D) culture (Geckil *et al.*, 2010). However, in an *in vivo* condition, neuronal cells are surrounded by other cells and extracellular matrix (ECM) and form highly organized neuronal networks. For biomimetic measurements, cells should grow in as natural an environment as possible, and thus the development of a biomimetic 3D structure for neuronal cells is crucial. Although 2D neuronal model systems have major advantages, such as biocompatibility, controllability, and observability, they have serious limitations in exhibiting the characteristics of *in vivo* systems (Yoo *et al.*, 2011). To gain a deeper comprehension of the neural systems, numerous *in vitro* approaches mimic several spatial-temporal cell extrinsic stimuli, often in the form of the 3D tissue culture. Culturing human derivatives (hESCs/hiPSCs) in 3D has opened up new opportunities for the exploration of the human development and regenerative medicine approaches, especially in the field of neurodegenerative diseases, neuronal differentiation, neurite formation, and spatial orientation in tissue-like cultures (Agholme *et al.*, 2010). Fundamental differences exist between cells cultured in monolayer or 3D structures. For example, when comparing 2D and 3D embryonic mesencephalon tissue, more cell death occurs in dissociated monolayer cultures, while 3D cultures in collagen gels survived to a larger extent (O'Connor *et al.*, 2000). A growing body of evidence reveals that the elements of a 2D environment could lead to change from gene expression, metabolism, and extracellular matrix composition to cellular functionality (Birgersdotter *et al.,* 2005). In contrast, 3D-cultured cells are more reflective of *in vivo* cellular responses (Antoni *et al.*, 2015).

3D *in vitro* cultures try to mimic the *in vivo* cell environments by placing cells from immortalized cell lines, such as stem cells or explants, within the hydrogel or specialized matrices. The more similar a cell culture system is to native tissue, the greater the potential for representative results. To date, the 3D cell culture models have exhibited features that are closer to complex *in vivo* conditions. It is also known that the 3D models have proven to be more practical for translating the findings to *in vivo* applications (Bouet *et al.*, 2015). Given the importance of cell-to-cell interactions in the human brain, various laboratories have begun characterizing 3D brain cell culture models. As a result, the 3D model has the potential for blocking the release of certain neurotrophic factors and interfering with cell adhesion molecules. Additionally, studies using iPSCs from RETT patients in a classical 2D adherent culture have revealed reduced neurite outgrowth and synapse number, distorted calcium transients and spontaneous postsynaptic currents while, in 3D, the model allowed for the creation of layered

architectures thereby accelerating maturation of neurons from human iPSC-derived neuronal progenitor cells (NPCs), yielding electrophysiologically active neurons within 3 weeks (Marchetto *et al.*, 2010).

1.4 Objectives

The scientific **aim** of this study was to identify those parameters, which influence the *in vitro* differentiation of pluripotent cells. These factors are important in early developmental stages and act on gene/metabolic pathways, driving the lineage commitment of stem cells.
Our **hypothesis** behind the study was that the mouse, as a model system, could mimic the early event and lineage decisions of mammals, therefore the developed differentiation protocol can be implemented in human pluripotent stem cell directly, with minor modifications. Hence, differentiation of pluripotent stem cell of mouse and human were investigated by cross-comparisons during lineage-specific differentiation events.

The key questions behind the study:

(1) Which mechanisms and metabolic pathways are involved in early developmental decisions of *in vitro* stem cell differentiation?

(2) Are there any major differences among the neuroectodermal lineage differentiation pathways of pluripotent cells of the two species?

(3) Is it possible to establish a reproducible and homogeneous differentiation method, which may allow the development of standardized protocols for human applications?

Specific objectives of the research were:

(1) Differentiate mouse pluripotent stem cells into the neuroectodermal lineage.

(2) Study neuroectodermal lineage commitment and understand the limitations of *in vitro* pluripotent stem cell differentiation.

(3) Establish a lineage specific stem cell differentiation model, which eventually can mimic tissue development or disease pathology *in vitro*.

(4) Adapt the mouse model to human stem cells and test a lineage-specific cellular system *in vitro*.

(5) Finally, establish a new, robust *in vitro* protocol for mouse and human neuroectodermal differentiation from induced pluripotent stem cells.

2. THE LITERATURE REVIEW

2.1 Stem cells and its characteristics

The term 'stem cell' was originated from the German word "Stammzellen" in the year 1868 by the eminent German biologist Ernst Haeckel. The concept of stem cells was first described in one of the earliest publications (Haeckel *et al.,* 1850). The history of stem cell science began in the 20th century where single cells from the bone marrow gave rise to different kinds of blood cells from both humans and rodents (Till and McCulloch, 1961; Thomson *et al.,* 1995).

Stem cells are unique cell types, which have the remarkable potential to develop into many different cell types in the body during early life and growth. Stem cells are distinguished from other cell types by two important characteristics. Most importantly, they are unspecialized cells, which have the ability to replenish themselves throughout the cell division (self-renew), and secondly they can give rise to one or more specialized cell types through asymmetric cell divisions (differentiate). As they begin to differentiate, their differentiation potential becomes more restricted (described in Figure 1). Based on their pluripotency we can distinguish the following stem cell categories:

Totipotent stem cells (TSCs): TSCs have the largest versatility from all the other stem cell types. Especially in mammals, the fertilized egg, up to the 4-8 cell stage blastomeres (which varies species specifically) can be considered totipotent, meaning that they can give rise to an entire organism including the extraembryonic tissues (Petros *et al.,* 2011).

Pluripotent stem cells (PSCs): PSCs can give rise to most of the cell types, the somatic cells (the three germ layers) and germ cells. However, they are not capable of forming extra-embryonic tissues. Based on their origin there are different subtypes (1) Embryonic stem cells (ESCs); (Thomson *et al.,* 1998; Thomson *et al.,* 1995); (2) Embryonic germ cells (EGCs) (Thomson and Odorico, 2000; Turnpenny *et al.*, 2003) and (3) Embryonic carcinoma cells (ECSs) (Andrews *et al.*, 2005) and (4) the induced pluripotent stem cells (iPSCs;Takahashi and Yamanaka, 2006; Takahashi *et al.,* 2007); which we detail later.

Multipotent stem cells (MSCs): MSCs are also known as somatic or adult stem cells. They have been identified in various tissue sources such as muscle, bone marrow, adipose tissue, retina, pancreas, central nervous system, dental pulp, blood, intestine, skin. However, previously these cells were thought that they give rise only to limited tissue origin. But studies over the past years suggest that adult stem cells from some tissue might have the ability to differentiate into cell types from all three germ layer (Li *et al.*, 2013; Strzyz, 2016).

Oligopotent stem cells (OSCs): OSCs could differentiate lineage specifically. Examples such as lymphoid or myeloid cells (Kondo, 2010).

Unipotent stem cells (USCs): USCs can be differentiated into single mature cell type, but they have the property to self-renew. Examples such as muscle stem cells (Brack and Rando, 2012; Patsch *et al.*, 2015).

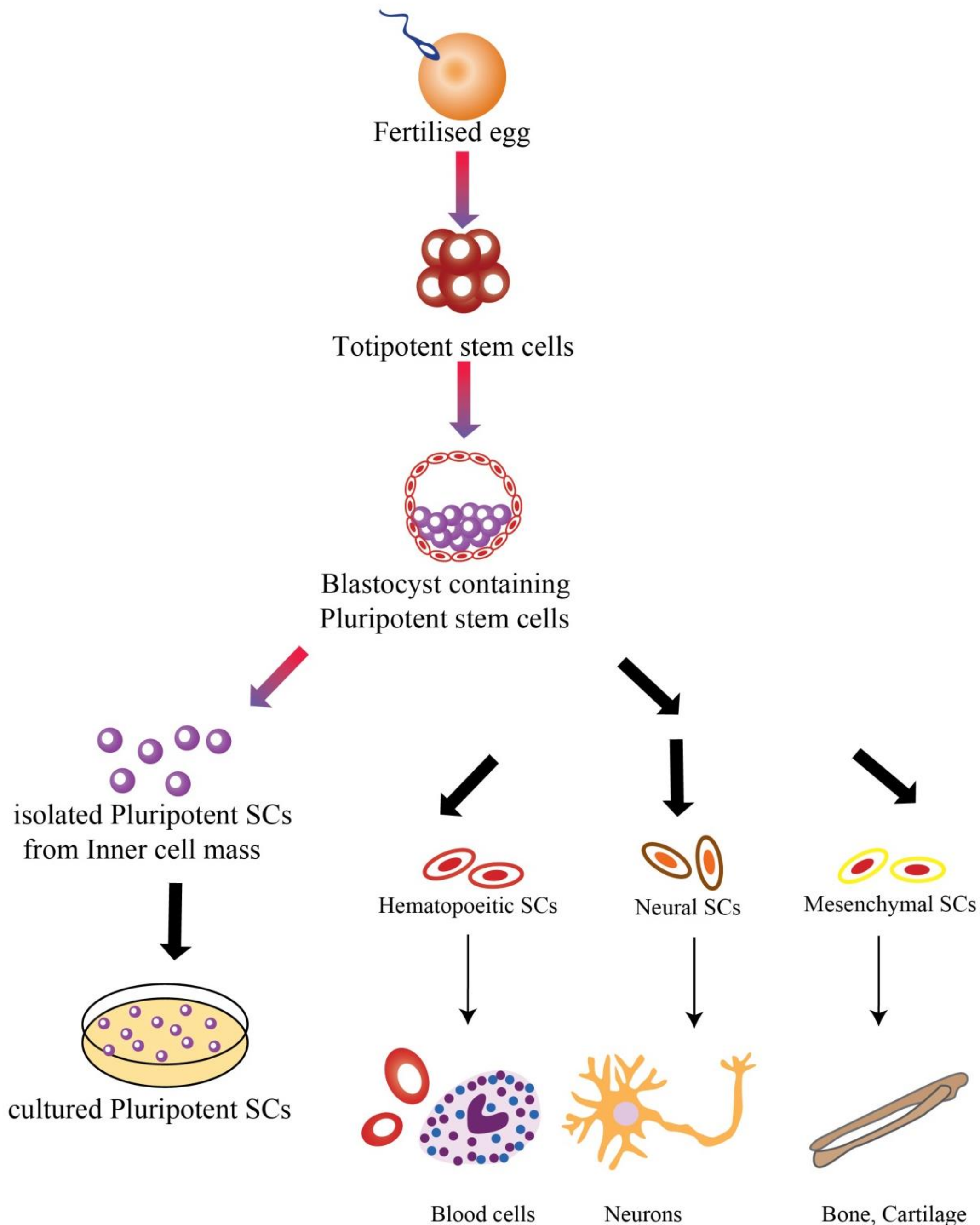

Figure 1. Pluripotent stem cells, such as embryonic stem cells (ESCs) originate from the inner cell mass (ICM) of the blastocyst. The ICM cells can differentiate into any tissue type of the body excluding extra embryonic lineages. Adapted from (Chaudry, 2004).

As we mentioned, above, stem cells can be clustered based on their tissue origin as well, into the following major subtypes.

Embryonic Stem Cells (ESCs)**:** as their name shows, they are derived from preimplantation stage embryos, usually from inner cell mass of blastocyst stage embryos. During the early embryonic development, the cells remain relatively undifferentiated and possess the ability to differentiate into almost any tissue type within the body. ESCs have two important characteristics: self-renewal and pluripotency (Kaufman *et al.*, 1983) which is used widely in *in vitro* applications (Boheler *et al.*, 2002).

Fetal Stem Cells (FSCs): FSCs are embryonic cell types found in the organs of the fetus. Fetal stem cells can be isolated from fetal blood as well as bone marrow; additionally, they can be isolated from liver and kidney of the fetal organs (O'Donoghue and Fisk, 2004). Fetal blood is a rich source of hematopoietic stem cells, which proliferate more rapidly than those in cord blood or adult bone marrow (Guillot *et al.*, 2006). Like adult stem cells, fetal stem cells are tissue-specific and generate the mature cell types within the particular tissue or organ in which they are found. The classification of fetal stem cells is currently unclear.

Cord Blood Stem Cells (CSCs): At birth, the blood in the umbilical cord is rich in blood-forming stem cells. They are classified as "multipotent stem cells" (Lee *et al.*, 2004; Rogers and Casper, 2004; Musina *et al.*, 2007) that can differentiate into certain cell types. The applications of cord blood are similar to those of adult bone marrow and are currently used to treat diseases and conditions of the blood and to restore the blood system after treatment for specific cancers or to restore immune system conditions such as leukemia and sickle cell anemia. In the recent years , umbilical cord blood transplantation (UCBT) is increasingly used for a variety of malignant and benign hematological and other diseases (Chou *et al.*, 2010; Forraz and Mcguckin, 2011; Ballen *et al.*, 2014).

Adult Stem Cells (ASCs): ASCs are also known as somatic SCs. These are undifferentiated cells, which are found in tissues or organs of an adult mammalian. Their function is to maintain and repair tissues in a living organism. Adult stem cell treatments have been successfully used for many years to treat leukemia and related bone/ blood cancers through bone marrow transplants (Sollazzo *et al.*, 2011). Adult bone marrow contains at least two types of stem cells: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) (Oswald *et al.*, 2004).

Induced Pluripotent Stem Cells (iPSCs): iPSCs are directly generated from adult cells. These cells are reprogrammed to a pluripotent state by the introduction of reprogramming factors (Takahashi and Yamanaka, 2006; Gurdon *et al.*, 1971). This technology gained importance in the field of disease modeling and drug screening by replacing animal models. The technology and most important features of iPSCs will be described in later sections.

2.2 Embryonic stem cells (ESCs)

The first mouse ESCs were isolated from the intact mouse pre-implantation blastocysts (M J Evans and Kaufman, 1981) and the entire ICM of the early-stage mouse embryo was cultured in medium conditioned by an established teratocarcinoma stem cell line (Martin, 1981). The first successful tetraploid complementation was documented by (Nagy *et al.,* 1993). The cells that arise from blastocysts are pluripotent in state and at this stage are referred to as blastocyst embryonic stem cells (Thomson *et al.,* 1998). Numerous mouse ESC lines have been subsequently generated from different mouse strains with various derivation efficiency and approaches (Eakin and Hadjantonakis, 2006; Lau *et al.*, 2016). The capacity of mouse ESCs to form a teratoma *in vivo* was demonstrated by injection of mouse ESCs into nude mice, where the formed teratoma consist tissues from the three primary germ layers (Bjorklund *et al.*, 2002). Apart from cell differentiation in a teratoma, mouse ESCs can also contribute to embryo development and be part of the tissues of an embryo through chimeras (Eakin and Hadjantonakis, 2006). Moreover they can differentiate into the germ cells of the chimera animal, therefore provides an effective model to generate germ-line chimeras (Nagy *et al.*, 1993), which

are useful for gene knock-out and precise genome modification (for example homologous recombination or CRISPR/Cas9 gene editing) to study specific genes (Zwaka and Thomson, 2003).

Mouse ESCs are typically grow in compact colonies with tight, rounded, domed morphology when cultured on mitotically inactivated mouse embryonic fibroblast (MEFs) in the presence of leukemia inhibitory factor (LIF) or BMP4 which are needed to maintain the pluripotent state of stem cells (Martin, 1981; Smith, 2001; Ying *et al.*, 2003). However, these techniques have several drawbacks, including the need for feeder cells and use of undefined media containing animal-derived components, such as serum. The culture of stem cells under undefined conditions can induce spontaneous differentiation and reduce reproducibility of experiments. Hence, over the years, various methods have been employed to address the apparent specific requirements of mouse ESCs and to improve methods for their derivation and culture. Especially the discovery that was demonstrated by Ying and colleagues, showed that inhibition of MEK/ERK and glycogen synthase kinase-3 (GSK3) signaling (also called as the “2i” condition) were together sufficient, combined with activation of Stat3 by LIF (2i/LIF), to promote the pluripotent ground state of emergent ESCs from mice (Ying *et al.*, 2008; Buehr *et al.*, 2008; Czechanski *et al.*, 2014).

The recent advances in hESC biology have generated great interest in the field of stem cell-based engineering, but issues regarding their safety must be overcome first. Human ESCs have been successfully derived from the different stages of human embryos: blastocyst, morula stage embryos, arrested blastocyst embryo and blastomeres (Thomson *et al.,* 1998; Pera *et al.,* 2000; Warmflash *et al.*, 2014). Also, they have also been derived from human somatic cell nuclear transfer (SCNT) embryos, termed human nuclear transfer ESCs (NT-ESCs) (Tachibana *et al.*, 2013). *Human ESCs* exhibit very large nuclei, a minimal amount of cytoplasm and few organelles which are similar to mouse ESCs (Adewumi *et al.*, 2007; Allegrucci and Young, 2007). In contrast, to mouse ESCs, human ESCs tend to form flatter and loose structure rather than a domed shaped colony (Verlinsky *et al.*, 2005; Cockburn and Rossant, 2010). Culturing hESCs in basic fibroblast growth factor (bFGF) can maintain self-renewal capacity of human stem cells. Other components to maintain stem cells characteristics are (i) feeder cells, conditioned medium, or cytokines, such as transforming growth factor beta (TGF), or WNT3A, (ii) fetal bovine serum (FBS) or serum replacement (iii) matrix, such as matrigel or fibronectin or laminin (Hanna *et al.*, 2010; McEwen *et al.*, 2013). Until recently hESC lines were derived in medium containing an animal product. The presence of xenograft in hESC culture media lead to the formation of toxic proteins, increase the risk of animal pathogens and the use of animal products complicate developmental studies. Therefore, it was important to grow hESCs in a defined medium without animal products (Rajala *et al.*, 2007).

Some of the potential applications of embryonic stem cells are in cardiovascular disease, spinal cord injuries, and glaucoma. A recent study by Shroff et al. showed that transplanted hESCs to the injury site of spinal cord injury patients improves body control, balance, sensation, and limbal movements (Shroff and Gupta, 2015). Additionally, ESCs can be directly differentiated into insulin secreting β-cells (marked with GLUT2, INS1, GCK, and PDX1) which can be achieved through PDX1 mediated epigenetic reprogramming (Salguero-Aranda *et al.*, 2016). In a nutshell, ESCs holds promise in regenerative medicine, however, currently, it is unclear how useful these cells will be in clinical applications due to the existing ethical concerns.

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2.3 Cell commitment and the Waddington, landscape model

To generate pluripotent stem cells and further clinically relevant cell types, it is important to understand the regulation of cell differentiation and the transcription factors, which drives it. Although iPSCs are a relatively new field of research, the foundation of this field was laid over 50 years ago.

Initially, it was believed that acquisition of cell fate could occur unidirectionally, from an immature or pluripotent to a mature or differentiated state and this idea has been depicted as a ball rolling down from the top of Waddington's 'mountain' to the bottom of a 'valley.' However, a series of landmark experiments showed that cell fate is flexible and reversible. It is now known that cells can, in fact, transition from a differentiated to a pluripotent state (depicted as climbing Waddington's hill) in the course of rejuvenation or reprogramming. The first experimental indications of this cellular plasticity were provided by approaches involving the transfer of somatic nuclei into an enucleated egg or fusion of a somatic cell with a pluripotent stem cell, which has shown that epigenetic program of the somatic genome can be erased and that cells can be rejuvenated to pluripotency. It has also been demonstrated that ectopic expression of tissue-specific transcription factors can convert a differentiated cell to a cell of another lineage, a process known as transdifferentiation (direct cell conversion) and depicted as moving from one valley to another valley across the ridge of Waddington's landscape, which is illustrated in Figure 2.

In 1957, Conrad Waddington described that mammalian development is unidirectional, which means that embryonic stem cells develop into a more mature differentiated state. He explained that stem cells are the top of a mountain and that they 'roll down' like marbles, becoming more differentiated cells (Waddington, 1956, 1957). During this time, it was believed that cells become specialized by deleting or inactivating unnecessary genetic information. Later, in 1962, John B. Gurdon showed for the first time by nuclear reprogramming that adult somatic cells could resort back into PSCs. He transferred a nucleus of a tadpole's somatic cell into an enucleated oocyte, indicating that factors in the oocyte cytoplasm can reprogram somatic nuclei to a pluripotent state and succeeded in obtaining a cloned frog (Gurdon *et al.*, 1971; Gurdon, Laskey and Reeves, 1975). This means, the cells did not lose the "information" during their differentiation, the "un-used" genes are just silenced, but can be reactivated upon proper stimuli.

In 2006, Yamanaka and co-workers created new ventures in disease modeling and regenerative medicine (Takahashi and Yamanaka, 2006). Their concept involves in combining the four selected transcription factors (TF) OCT3/4, SOX2, KLF4 and C-MYC to generate iPSC directly from mouse embryonic or adult fibroblast cultures by retroviral introduction of the four genes. The concept was later translated to human somatic cells (Takahashi *et al.,* 2007). Detail on genetic reprogramming will be explained in the next section.

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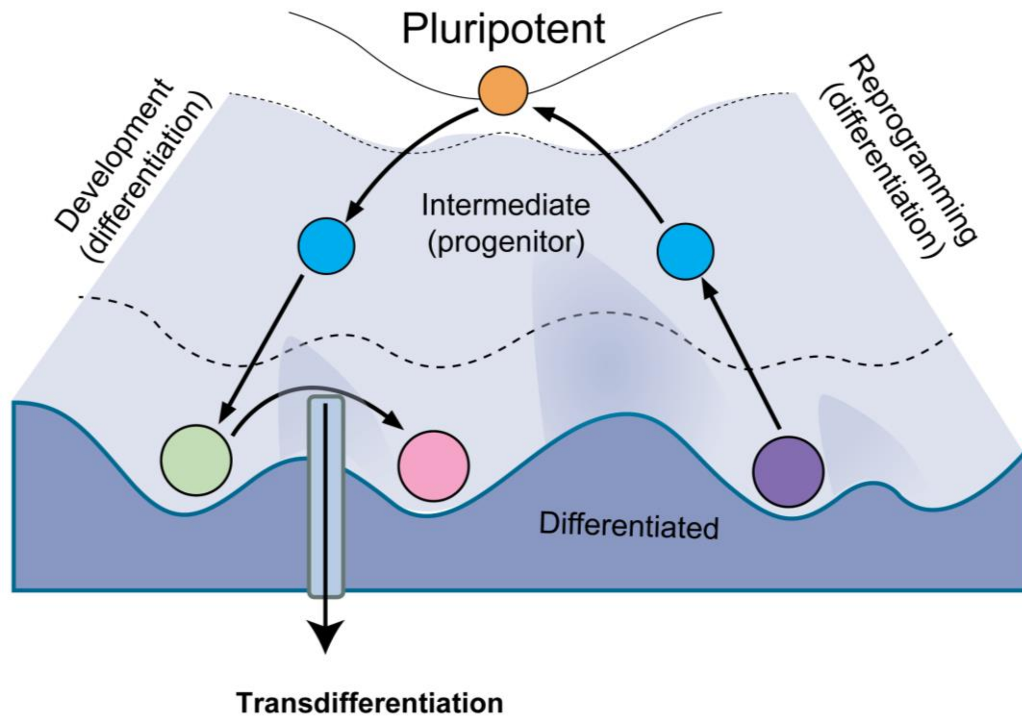

Figure 2. **Cell fate Plasticity**. The image is depicting Waddington's landscape. The figure was adapted from (Kazutoshi Takahashi and Shinya Yamanaka, 2016)

2.4 Genetic reprogramming

Genetic reprogramming is a technique where resetting the epigenome of a somatic cell to a pluripotent state occurs (Buganim *et al.,* 2013). Reprogramming can be achieved through the introduction of exogenous factors or so called as transcription factors, cell fusion or by somatic cell nuclear transfer (SCNT)(Jaenisch and Young, 2008). Somatic cloning may be used to generate multiple copies of genetically modified farm animals, to produce transgenic animals for pharmaceutical protein production, or to preserve endangered species. The first success of cloning an entire animal, Dolly (the sheep), from a differentiated adult mammary epithelial cell (Campbell *et al.*, 1996; Wilmut *et al.*, 1997) that created a revolution in modern science. Shortly, after that, this practice became an essential tool for studying gene function; genomic reprogramming in various species (Munsie *et al.*, 2000; Dinnyés *et al.*, 2002). So far, SCNT approach has been successfully performed in mouse, frog and human cells, several farm- and hobby animals, and endangered species (Lagutina *et al.*, 2013; Wilmut, Bai and Taylor, 2015). Despite the progress, the efficiency of nuclear transfer is still low; further investigation is needed to improve culture conditions and enhance the efficiency.

As we mentioned in section 2.1. The generation of induced pluripotent stem cells was first reported by Yamanaka's research group (Takahashi and Yamanaka, 2006; Takahashi *et al.,* 2007). In the original concept, they selected all those genes, which were known to drive pluripotency regulation and might be related to the stem cell stage. After that, they overexpressed the different combinations of genes in fibroblast cells to choose those combinations where the reprogramming happened, demonstrating that fibroblast can be transformed to a stem cell stage. Their method enables the reprogramming of somatic cells to pluripotent stem cells by the transfection of only four transcription factors, namely Oct3/4, Sox2,

c-Myc, and Klf4. The generated iPSC cells become indistinguishable from embryonic stem cells based on cell morphology, gene expression profile, and teratoma formation. However, the process is long and generates iPSCs that vary extensively in their developmental potential (Yamanaka and Blau, 2010). Until now there have been numerous reports describing the derivation of iPSCs in various species, such as mouse (Okita *et al.*, 2008), human (Takahashi *et al.,* 2007), rat (Li *et al.*, 2009), pig (Esteban *et al.*, 2009), cattle (Han *et al.*, 2011), or sheep (Sartori *et al.*, 2012). Recently human iPSCs have been generated from a range of patients including, Alzheimer's disease, Parkinson disease, Huntington disease, and Amyotrophic lateral sclerosis, which are very useful for studying *in vitro* the pathomechanisms of the diseases or cell replacement models and for drug discovery.

2.4.1 Role of transcription factors in the maintenance of pluripotency

In this section, we detail those transcription factors, which are important in the maintenance of pluripotency in mouse and human.

Oct4/OCT4: *Pou5f1* gene (is also known as *Oct3*, or *Oct4*) was first identified as an ESC-specific and germline-specific transcription factor (Zhang *et al.*, 2007). They form a trimeric complex with Sox2 on DNA and control the expression of a number of genes involved in embryonic development such as *YES1, FGF4, UTF1, and ZFP206.* *in vivo* ablation of *Oct4* in mouse embryo leads to a defect in the viability and development potential of the ICM (Kehler *et al.*, 2004). In humans, *Pou5F1* is one of the most studied genes in pluripotency research to determine the self-renewal capacity**.** It's most relevant roles are the (i) determination of growth factor signaling from stem cells of the embryo to the trophectoderm (TE); (ii) it regulates the cell fates of pluripotent cells; (iii) the repression leads to a loss of pluripotency and dedifferentiation to TE; (iv) it plays indirect role in regulating the FGF4 expression. This is important for the differentiation and maintenance of extra-embryonic endoderm from the TE (Avilion *et al.*, 2003).

Sox2/SOX2: *Sox2/SOX2*, is an high mobility group (HMG) box transcription factor, which has been shown to be central to the transcriptional network regulating pluripotency in mouse and human ESCs (Ginis *et al*., 2004). Sox2, together with other stem cells transcription factors, like *Oct4* and *Nanog,* they form a critical regulation network, which regulates transcription of other genes and is important in the development of ICM and TE (Niwa *et al.*, 2005). The transcription factor *Sox2/SOX2* is a key player in the maintenance of pluripotency and "stemness" both in human and mouse. It is also involved in regulating the expression of Fgf4/*FGF4* in both mouse and humans. In humans, SOX2 can be replaced by closely related SOX family members, SOX1 and SOX3, in the generation of iPSCs (Takahashi *et al.,* 2007), but not by more distant members, like SOX7 and SOX15 (Nakagawa *et al.*, 2008).

Klf4/*KLF4:* Krueppel-like factor 4 protein is a transcription factor found both in human and mouse. They act as an activator and/or a repressor. It plays an important role in maintaining embryonic stem cells, and in preventing their differentiation (Kim *et al.*, 2009). It is also required for establishing the barrier function of the skin and for postnatal maturation and maintenance of the ocular surface. It is involved in the differentiation of epithelial cells and may also function in skeletal and kidney development. Klf4/KLF4 also contributes to the down-regulation of p53/TP53 transcription (Rowland and Peeper, 2006). A study reported that the KLF4 was

overexpressed in pluripotent cells, which had a greater capacity to self-renew. It was shown that KLF4 overexpressed cells show higher levels of OCT3/4 with the conception that KLF4 promotes self-renewal (Papapetrou *et al.*, 2009).

cMyc/c-MYC: cMyc has been implicated in the maintenance of ESCs, and it was reported that ***cMyc*** works downstream from the LIF/STAT3 pathway (Cartwright *et al.*, 2005). Similarly, like other transcription factors *cMyc*/C-MYC plays an important role in cell growth, differentiation, proliferation and also self-renewal of stem cells both in human and mouse. They are often overexpressed in cancer cells. Expression of *c-MYC* can activate *β-CATENIN* (Hyun *et al.*, 2007).

Nanog/*NANOG:* *Nanog/NANOG* is another important homeobox transcription factor that is involved in the self-renewal of ESCs and is a critical factor for the maintenance of the undifferentiated state of PSCs. It was first identified by (Chambers *et al.,* 2003). *Nanog* is specifically expressed in pluripotent cells and plays an essential role in the maintenance of pluripotent mouse ESCs. Importantly, high level of *Nanog* allows mouse ESCs to self-renew in the absence of the extrinsic LIF and blocks primitive endoderm differentiation, suggesting that *Nanog* may be a major downstream effectors for extrinsic factor (Chambers *et al.*, 2003). The transcription factor *Nanog/NANOG* is present in pluripotent cells of both human and mouse cell lines but not in differentiated cells. Additionally, NANOG protein helps to propagate ESCs. With the cytokine stimulation of STAT3, NANOG can drive ESCs to self-renewal (Mitsui *et al.*, 2003). Besides these reprogramming factors, other genes such as *STAT3, LIN28, and β-catenin* have been shown to account for the long-term maintenance or proliferation of pluripotent cells.

Stat3/*STAT3:* *Stat3* gene knocks out in mice resulted in early embryonic lethality. S*tat3* deficient mouse embryos fail to develop beyond E.7 when gastrulation begins (Takeda *et al.*, 1997). Additionally, *Lif/Stat3* signaling is required for the maturation of mouse iPSC reprogramming. STAT3 is dependent in both human and mouse cells to sustain self-renewal capacity. However more significantly STAT3 activation has to occur in the presence of p300/CBP coactivator complex to initiate the self-renewal of pluripotent cells (Freeman, 2010). STAT3 is activated through the tyrosine phosphorylation cascade after ligand binding with the growth factor receptor complex and cytokine receptor-kinase complex (Moon *et al.*, 2002).

Ctnnbl1/CTNNB1: Catenin ***beta-1 is also known as β-catenin.*** Along with WNT-signal-transduction pathway, they play a key role in mESCs and hESCs for the cell-fate determinant. Uncontrolled accumulation of Catenin beta-1 can result in developmental defects and tumorigenesis in humans (Kielman *et al.*, 2002).

Lin28/LIN28: LIN28 is an RNA-binding protein that is recognized for its roles in promoting pluripotency via regulation of the microRNA let-7. In mouse and humans, *LIN28* is expressed early during development and in undifferentiated tissues. Despite its roles in pluripotent cells, *LIN28* has also been shown for proper differentiation (Faas *et al.*, 2013).

2.4.2 Important signaling pathways which underlie pluripotency

In mammals, OCT4, NANOG, and SOX2 are the key transcription factors that are central to the transcriptional regulatory hierarchy and play essential roles in maintaining pluripotency and self-renewal of ESCs, as detailed in the previous section. These factors can activate genes required

for cell survival and proliferation while repressing the activity of differentiation-associated genes. In addition to this triad, there have been other transcription factors that have been shown to function interdependently and form a large gene regulatory network in pluripotency. The most important and highly cited ones are explained below in **Table 1**.

Table 1: Signaling pathways in mouse and human pluripotent cells. The table was adapted from (Schnerch *et al.,* 2010).

Pathways	Gene	Functions
Mouse		
LIF	*Lif*	-IL-6 families of cytokines. -Required for blastocyst implantation - Affects the differentiation, survival, and proliferation of a wide variety of cells in the adult and the embryo
	Lifr	LIF action appears to be mediated through a high-affinity receptor complex composed of a low-affinity LIF binding chain (LIF receptor) and a high-affinity converter subunit, gp130. -Polyfunctional cytokine that is involved in cellular differentiation, proliferation and survival in the adult and the embryo.
	Stat3	-Activated via JAK/STAT3 singling pathway to maintain pluripotency.
	Jak	
BMP	*Bmp4*	-Maintains the undifferentiated state of via inhibition of both ERK and p38.
Human		
FGF	*FGF2 or FGFB*	-FGF signaling is essential to the self renewal of human ESCs. -Plays an important role in the regulation of cell survival, cell division, angiogenesis, cell differentiation and cell migration. -Functions as potent mitogen in vitro. Can induce angiogenesis
TGF-β	*TGFB1 or TGFB*	-TGF-β/Actin/Nodal is a branch of TGF-β superfamily. -Activin/Nodal binds itself to the TGF-β ligand resulting in the phosphorylation of SMAD2 and SMAD3. -TGF-β combined with LIF and bFGF can prolong undifferentiated propagation of human ESCs.

2.4.3 Pluripotency states: Naïve and Prime

Pluripotent stem cells are classified into two distinct states naïve and primed stem cells (Leehee *et al.*, 2016). In pre-implantation embryos, pluripotent stem cells are referred to as a naïve state (ground-state), while the prime state is established in the epiblast of the mature blastocyst and may be captured *in vitro* in the form of fetal stem cells (Li and Ding, 2011; Huang *et al.,* 2014). Although rodent cells can exist in both primed and naïve pluripotent states, establishing a naïve state in human cells has been difficult to obtain (Nichols and Smith, 2009; Gafni *et al.*, 2013). These two stages are different from each other regarding morphology, gene expression profile and DNA methylation profile, but both can differentiate into the cells of the three germ layers. The naïve state pluripotent stem cells represent the epiblast in the preimplantation embryos e.g. mouse ESCs and mouse iPSCs. On the other hand, mouse epiblast stems cells (mEpiSC), human

iPSCs and human ESCs that represent the post-implantation embryo are thought to represent the primed state (Davidson *et al.,* 2015; Leehee *et al.*, 2016).

2.4.4 Generation of induced pluripotent stem cells

The generation and use of iPSCs have become an attractive strategy for potential clinical applications such as disease modeling, cell-based therapy and drug screening purposes due to their potential to differentiate into any cell type of interest. A variety of methods have been reported to reprogram somatic cells into PSCs (Figure 3), including the integration based and non-integration based methods. Integration based methods include retroviral, lentiviral and inducible lentiviral methods.

The process involves in reprogramming somatic cells towards pluripotent state without integrating pluripotency factors into the genome, which includes adenovirus (Zhou and Freed, 2009), Sendai virus-mediated (Ban *et al.*, 2011), episomal plasmid (Okita *et al.*, 2011), protein (Kim *et al.*, 2009), small molecules (Hou *et al.*, 2013) and miRNA (Lin *et al.*, 2008) methods. Both human and mouse stable iPSCs cells were successfully generated without genomic integration (Jincho *et al.*, 2010; Zhou and Zeng, 2013). The different somatic cell reprogramming methods are also detailed in Table 2.

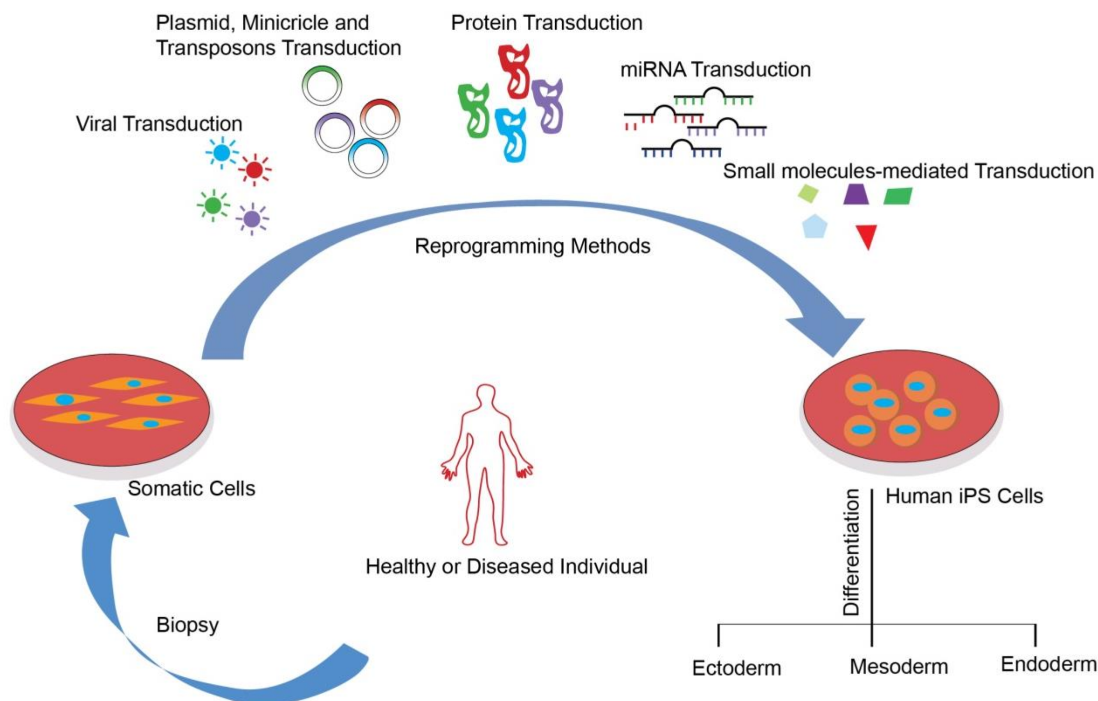

Figure 3. **Different methods of genetic reprogramming.** The somatic cells can be directly reprogrammed into human iPSCs by insertion of common iPSC reprogramming factors via various methods: viral transduction, plasmid, minicircle and transposon transduction, protein and microRNA transduction methods, and small molecule-mediated transduction. The human iPSCs thus obtained have the potential to differentiate into any cell type of the human body via multiple lineages: ectoderm, endoderm, and mesoderm. The picture was adapted from (Dash *et al.*, 2015). To determine the degree of success garnered by reprogramming, we must explore the set of assays that were developed to assess the key characteristic of ES cells: pluripotency. Based on the existing efficiency dataset we have depicted the details in Table 2.

2.4.5 The use of iPSCs in regenerative medicine and disease modeling

Neurodegenerative diseases (NDs) are described as pathological conditions in which primarily neurons degenerate and lose their functionality. Such loss of functionality results in apoptosis and culminates in severe atrophy of the affected patient brain regions(Gitler *et al*., 2017). Pathogenesis of these diseases is complex, and the underlying mechanisms remain to be elucidated. The generation of patient-specific iPSC has opened up the possibility to generate *in vitro* disease models, which can be differentiated into any given cell type and offer the possibility to model disease, uncover novel mechanisms, and test potential therapeutics *in vitro* using patient-derived cells (Bahmad *et al*., 2017). These models not only appealing regarding understanding early pathology before the onset of symptoms in specific diseases but also offers the opportunity to identify modes of intervention, which could be beneficial in a variety of NDs (Pen and Jensen, 2016). Moreover, the advent of the CRISPR-Cas9 gene technology has improved the efficiency in genome editing and accelerated the generation of isogenic controls that retain the genetic background of the patients and makes precise genotype and phenotype correlations possible. (One of the databases, where an updated list can be found is http://www.informatics.jax.org/humanDisease.shtml).

The use of iPSCs for disease modeling is based on the fact that these cells are capable of self-renewing and that these cells can differentiate into all cell types of the human body that can be utilized for disease models. First Lee *et al*. used iPSCs for the modeling of pathogenesis in Familial Dysautonomia (Lee *et al*., 2009). Since then, there have been many cases in which iPSCs have been used in studying various mechanisms that play a role in different diseases. One of the most common iPSCs disease models that have been reported is the Parkinson's disease (PD). PD is a very common neurodegenerative disease, in which, dopaminergic neurons of *substantia nigra* (a structure in midbrain) get lost, and formation of Lewy's bodies (inclusions in the cytoplasm of neurons all over the body) occurs. Treatment of this disease had not been possible due to the time at which PD gets clinically manifested, the neurons have already been lost, which makes it very difficult to be able to study the underlying mechanisms of PD to develop a treatment of it. In such a situation, iPSCs can be used, and experiments have been carried out in this aspect. Nguyen *et al*. studied G2019S mutation in *LRRK2* (Leucine Rich Repeat Kinase2) gene. This mutation has been reported in cases of sporadic and familial PD. In this study, their results demonstrated that G2019S mutation iPSCs were able to differentiate into dopaminergic neurons and showed increased expression of key oxidative stress response genes and α-synuclein protein (Nguyen *et al*., 2011). Similarly, a different group has also worked for the generation of iPSCs in PD. Devine *et al.* developed iPSCs from fibroblasts taken from a PD affected person possessing triplication of Synuclein Gene by the transduction of four basic transcription factors. Their studies established a system to reduce the levels of α-synuclein. These iPSCs were then directed to differentiate into dopaminergic neurons *in vitro* for the study of PD (Devine *et al.,* 2011).

In the case of regenerative medicine, the injured or degenerated tissues are repaired by the generation of those tissues with the help of iPSCs in the laboratory and then transplanting them to the site of injury or degeneration. One such example is Retinitis pigmentosa (RP) where eye's retina degeneration causes impaired vision. For the treatment of RP, iPSCs were generated from the patient suffering from the disease which was then shown to differentiate into rod

photoreceptor cells (Yoshida *et al.*, 2014). Until now several clinical trials have been conducted for the treatment of advanced dry Age-related macular degeneration (AMD) by using retinal pigment epithelium (RPE) cell suspensions derived from embryonic stem cells (Schwartz *et al.*, 2015; Song *et al.*, 2015). One of the most interesting studies by Takahashi M *et al.* in 2014 showed that iPSCs generated from skin fibroblasts obtained from two patients with advanced neovascular AMD were differentiated into RPE cells. Most recently the same team showed that their transplanted RPE cells remained intact without causing metastatic tumors (Mandai *et al.*, 2017). The differentiation was considered successful, however, has yet to be sufficiently adapted for clinical use.

Table 2: Methods for reprogramming somatic cells into iPSCs. The table was adapted from (Robinton and Daley, 2012).

Vector Type		**Cell type**	**Factors**	**Efficiency in %**	**Advantages**	**Disadvantages**
Integrating	Retroviral	Fibroblast, keratinocytes, blood cells, adipose cells, stromal cells, liver cells	OSKM, OSK, OSK+VPs or OS+VPA	~0.001-1	Reasonably efficient	Slower kinetics
	Lentiviral	Fibroblast	OSKM	~0.1-1.1	Reasonably efficient and transducers dividing and non-dividing cells	Incomplete proviral silencing
	Inducible Lentiviral	Fibroblast, keratinocytes, blood cells	OSKM or OSKMN	~0.1-2	Efficient and controlled expression of factors	Requires transactivator expression
Excisable	Transposon	Fibroblast	OSKM	~0.1	Reasonably efficient and no genomic integration	Labor intensive screening
	LoxP-flanked lentiviral	Fibroblast	OSK	~0.1-1	Reasonably efficient and no genomic integration	Labor intensive screening and Lox-P site remain in the genome
Non-Integrating	Adenovirus	Fibroblast and Liver cells	OSKM	~0.001	No genomic integration	Low efficiency
	Plasmid	Fibroblast	OSNL	~0.001	Occasional genomic integration	Low efficiency
DNA Free	Sendai virus	Fibroblast	OSKM	~1	No genomic integration	Sequence sensitive RNA replicates
	Protein	Fibroblast	OS	~0.001	No genomic integration	Low efficiency, short half-life, more protein for applications
	Modified RNA	Fibroblast	OSKM or OSKML+VPA	~1-4.4	No genomic integration	Requires multiple rounds of Transfection.
	Micro RNA	Adipose stromal cells and dermal fibroblast	miR-200c miR-302s miR-369s	~0.1	Faster reprogramming methods	Low efficient

O, Oct4; S, Sox2; N, Nanog; K, KLF4; M, c-Myc; L, LIN28, VPA, valproic acid; VPs, viral particles.

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2.5 Lineage commitment: the neuronal development

Lineage commitment is a process at which the cells becomes restricted irreversibly to one particular fate and loses the potential to differentiate into other cell types (Nimmo *et al.,* 2015). One of the central questions in developmental biology is that how differentiated cell types are committed to a specific cell fate. The process of commitment can be divided into two stages. The first stage is called the specification where the cell fate is said to be specified when it is capable of differentiating autonomously when placed in a specific microenvironment such as a petri dish. The second stage of the commitment is called determinant where the cells or tissue is capable of differentiating autonomously even when placed into another region of the embryo (Gont *et al.*, 1993). Indeed it is also possible to induce it by the addition of growth factors; the cells can be a commitment towards certain cell specific lineages such as neurons, astrocytes, and oligodendrocytes in the case or neuronal lineage.

During embryogenesis, the central nervous system (CNS) develops from neural progenitor cells (NPC) within the ectodermal germ layer (Gont *et al.*, 1993). NPCs are usually found in the proliferative zone, which includes the neural plate (ventricular zone (VZ) and subventricular zone (SVZ)). These cells are responsible for the formation of neurons, astrocytes, and oligodendrocytes (Tang *et al.*, 2015). The formation of the CNS is initiated by a process called neurulation. Neurulation in humans starts at the end of the 3rd week of gestation and overlaps with the completion of gastrulation whereas in the mouse by the end of first week of gestation (Muñoz-Sanjuán and Brivanlou, 2002). Neurulation is induced by the activation and inhibition of different genes within the epiblast and thus resulting in neural tube formation (Stiles and Jernigan, 2010). The anterior part of the neural tube gives rise to the whole part of the brain containing the forebrain, midbrain and hindbrain neurons whereas the posterior part of the neural tube gives rise to the spinal cord (Stiles and Jernigan, 2010). Failure of these opening to close contributes a major class of neural abnormalities (neural tube defects) (Mitchell *et al.,* 2004; Bergström and Forsberg-Nilsson, 2012).

The formation of neural tube involves the inhibition of transforming growth factor beta (TGF-β) and bone morphogen protein (BMP) signaling, followed by anterior-posterior (A-P) axis and dorsal-ventral (D-V) axis patterning (Sakai, 1989; O'Rahilly and Muller, 1994). Forces generated by the surface epithelium as it expands towards the dorsal midline cause elevation of the neural folds and ultimately, closure of the neural tube. Neurulation in mouse starts very rapidly than in humans. Neurulation is induced by the activation and inhibition of different genes within the epiblast and thus resulting in neural tube formation (Greene and Copp, 2009). The process of neurulation has been depicted in Figure 4.

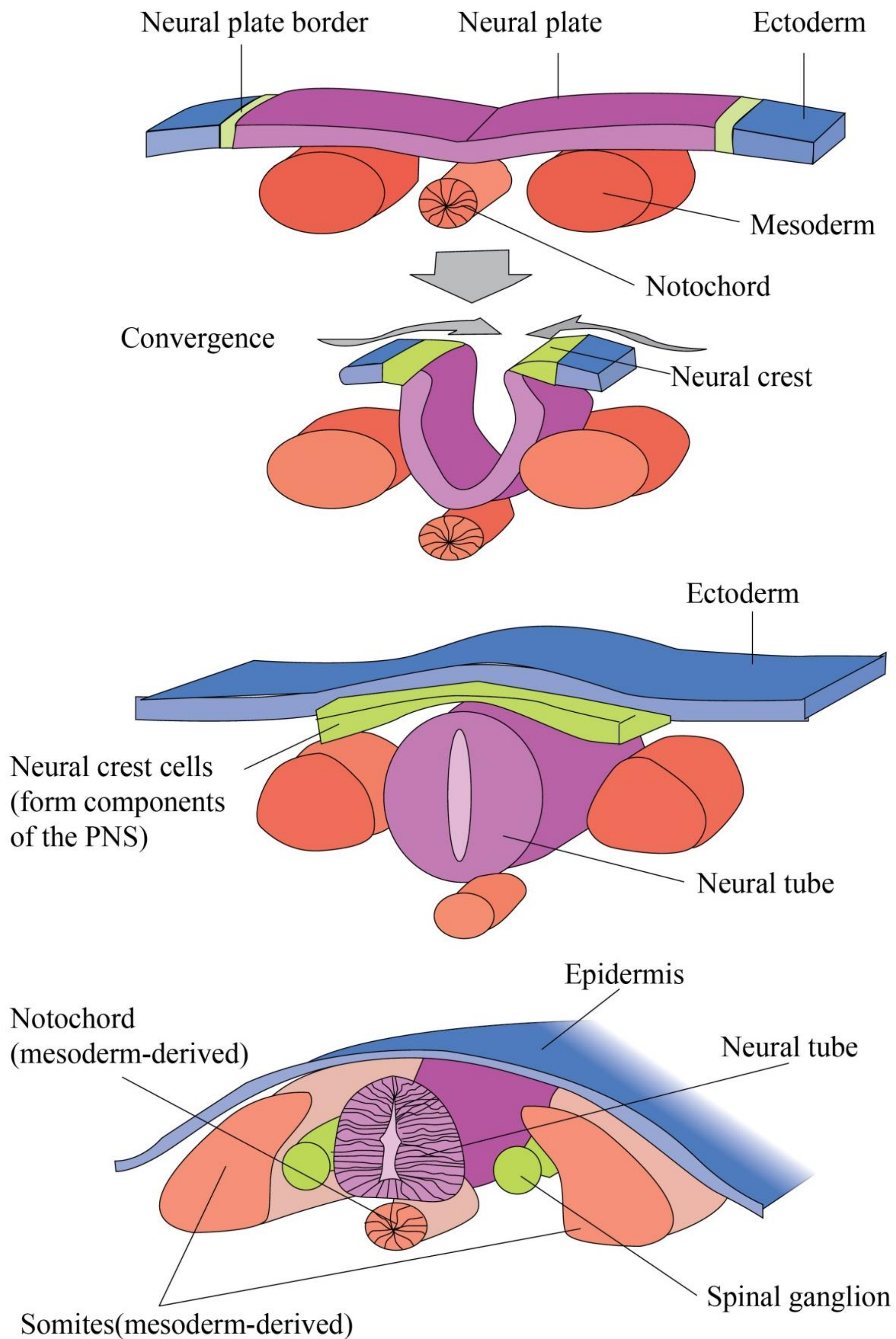

Figure 4. Border induction and neurulation: The neural plate is induced by neural inductive signals secreted by surrounding cells. During neurulation, the neural folds elevate, invaginate and pinch off from the surface to form a hollow tube resulting in forming the neural tube. Neural crest cells are established at the periphery of the non-neural ectoderm and the dorsal neural tube. Ultimately, these neural crest cells would migrate out and differentiate into specific cells types. Picture of neurulation adapted from (Gammill and Fraser, 2003). Failure or incomplete closing of the neural tube during neurulation results in a developmental congenital disorder called 'Spina bifida.'

Differentiation of neural stem cells *in vitro* will occur simply through the withdrawal of the mitogen. The commitment, or differentiation, into certain cell lineages such as neurons, astrocytes, and oligodendrocytes is also possible by adding various growth factors. For example, **FGF** signaling acts through activation of the extracellular signal-regulated kinase (ERK1/2) pathway, resulting in transcriptional activation of target genes. Blocking FGF signaling in ESCs virtually abolishes their neuroectodermal commitment (Suter and Krause, 2008). Likewise, **BMP** antagonist (noggin, chordin, follistatin) are produced by the notochord; they promote the formation of neuroectoderm (Liem *et al.*, 1995). **WNT–β-catenin** signaling also plays an important role during early neurogenesis. When active, β-catenin allows LEF/TCF transcription factors to be activated and induce gene expression (Valenta *et al.,* 2012). Finally, **Notch** signaling plays an important role in early neural induction, according to studies in the chick and the fruit-fly model; this is also corroborated by data in murine and human ESCs (Louvi and Artavanis-Tsakonas, 2006). In mammals, four different types of Notch receptors are described. Upon binding to the DSL (Delta, Serrate, and Lag-2) family of ligand, Notch receptors are cleaved in their transmembranous portion. During early neurogenesis, this cleaved part of Notch, also called the Notch intracellular cytoplasmic domain, will activate expression of members of the HES family of transcription factors, which will further regulate the fate of neuronal progenitors (Lundkvist and Lendahl, 2001; Carlén *et al.*, 2009). Therefore, a coordinated interplay between activation (FGF2, Notch, and WNT-β-catenin) and inhibition (BMP and WNT–β-catenin) of several signaling pathways is crucial for proper neurogenesis to be initiated.

2.6 Generation of different neuronal cells *in vitro*

The brain has enormously complex cellular diversity and connectivity that are fundamental for the neural functions. To uncover the pathogenesis of both early and late onset neurodegenerative disease it would be most conclusive to perform analyses on the neuronal subtypes which the disease mainly affects. The representation and proportions of various neuronal subtypes vary depending on the brain area. Different subtypes of neurons could be generated from iPSCs via initiating neural induction by inhibition of dual SMAD pathway, subsequently regionalization of these cells with the activation of neural patterning pathways using D-V patterning factors (BMPs and sonic hedgehog (SHH) signaling) and A-P patterning factors (retinoic acid (RA), FGFs and WNTs signaling) (Figure 5.) Several studies have clearly demonstrated the dual SMAD inhibition of BMP and TGF-ß signaling in PSCs promote forebrain identity.

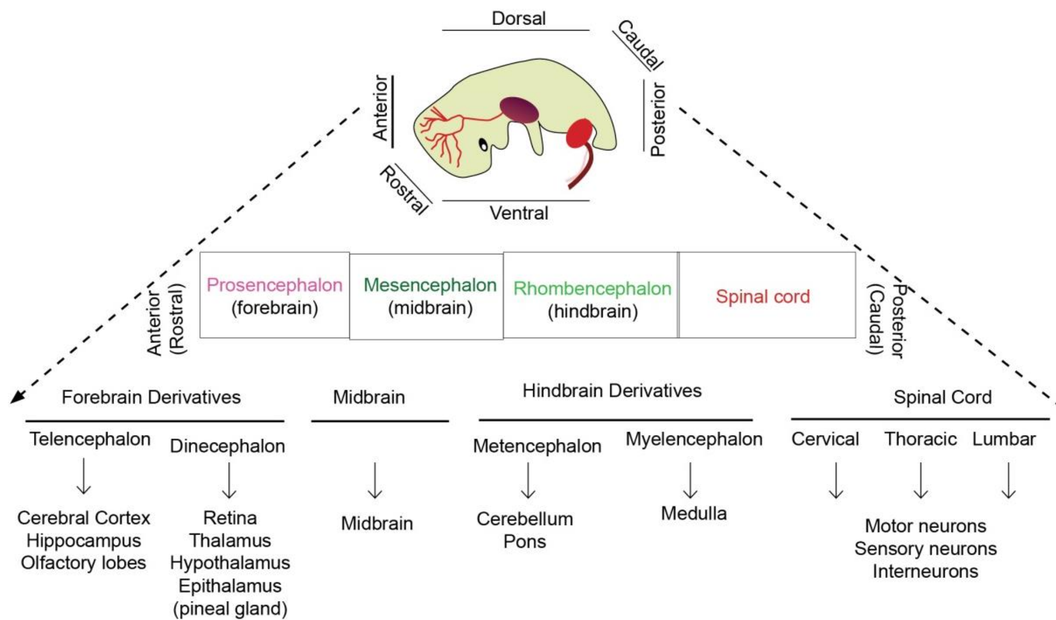

Figure 5. A depiction of vertebrate nervous system regional organization and generation of neuronal subtypes. This picture was adapted from (Petros *et al.,* 2011).

2.6.1 Dual SMAD pathway

The development and specification of the neural cells are closely linked to SMAD proteins, which play a central role by transducing extracellular signals from TGFβ and BMP signaling. Moreover, it seems they have a role in the pathomechanism of neurological diseases as well. SMADs are intercellular proteins that transduce these extracellular signals to the nucleus for gene regulation (Ueberham and Arendt, 2013). In general, signaling is initiated with ligand-induced oligomerization of serine/threonine receptor kinases and phosphorylation of the cytoplasmic signaling molecules SMAD2 and SMAD3 for the TGF-β/Activin pathway, or SMAD1/5/9 for the bone morphogenetic protein (BMP) pathway. Carboxyl-terminal phosphorylation of SMADs by activated receptors results in their partnering with the common signaling transducer SMAD4 and translocation to the nucleus. Activated SMADs regulate diverse biological effects by partnering with transcription factors resulting in cell-state-specific modulation of transcription. For instance, in the developing brain, the neural stem cell is generated by radial glia cells that can produce both glial cells and neurons. While TGFβ promoted differentiation of radial glia into astrocytes is mainly regulated by activation of MAPK signaling, neurogenesis is controlled by the SMAD2 and SMAD3 activity and PI3K activity (Stipursky *et al.,* 2012).

To achieve neuroectodermal fate, TGFβ and BMP signaling need to be inhibited (Massagué, 2012; Gámez *et al.,* 2013). This inhibition is facilitated by antagonists and inhibitors. Members of the TGFβ family (Nodal and Activin) binds to the TGFβ ligand, resulting in the activation of TGFβ signaling pathway. Activation of TGFβ leads to phosphorylation of SMAD2 and SMAD3, which translocate the phosphorylated SMADs into the nucleus, and thus induces mesodermal transcription factor. While BMP protein (BMP2, BMP4, and BMP7) binds to BMP ligand, causing the activation of BMP pathway. Likewise, activation of BMP pathway leads to phosphorylation of SMAD1, SMAD5, SMAD8, and SMAD9, which translocate the

phosphorylated SMADs into the nucleus, and thus induces epidermal transcription factor. Dual inhibition of BMP and TGFβ pathways with antagonists would result in neuroectodermal fate commitment (Oshimori and Fuchs, 2012; Kandasamy *et al.*, 2014). Not only proteins, but small molecules can also be used to inhibit the signaling: TGFβ can be inhibited by SB431542, while BMP can be inhibited by LDN193189 (Vogt *et al.,* 2011; Cai *et al.*, 2013).

2.6.2 Differentiation of forebrain glutamatergic, GABA and cholinergic neurons in vitro

NPC are generated with a default anterior fate commitment that can be sequentially differentiated into six distinct layers of cortical neurons recapitulating the corticogenesis *in vivo* (Shi *et al.,* 2012; Espuny-Camacho *et al.*, 2013). Deeper cortical layer markers (such as TBR1 and CTIP2) are the first, which can be detected after the initiation of the terminal differentiation followed by upper layer markers (SATB2, CUX1, CUX2, and BRN2). Shi *et al* produced almost 100% glutamatergic neurons from human PSCs with the expression of vesicular glutamate transporter 1 (vGLUT1) (Shi *et al.,* 2012) while Bissonnette *et al*. succeeded in the generation of Choline Acetyltransferase (ChAt) and vesicular acetylcholine transporter (VAChT) secreting neurons by exposing human iPSCs to posterior patterning factor retinoic acid (RA). Additionally, co-culturing NKX2.1 positive progenitor cells with human ESCs-derived astrocytes and treatment with nerve growth factor, resulted in the comparable generation of functional cholinergic and GABAergic neurons (Bissonnette *et al.*, 2011)

2.6.3 Differentiation of Spinal cord cells

During spinal cord development, several distinct neuronal subtypes are generated by the interaction of opposing morphogenetic gradients along the D-V axis of the neural tube, which establish a matrix of positional identities that in turn permit discrete precursor domains to emerge (Ericson *et al.*, 1996). For instance, motor neuron generation depends on two critical temporally distinct phases of SHH signaling (Gorris *et al.*, 2015; Zirra *et al.*, 2016): an early period, where it induces neural plate precursor cells to become ventralized, and a late period, where SHH drives the differentiation of ventralized precursors into motor neurons, at which point there is a concentration-dependent specification of ventral precursors into motor neurons or interneuron (Faravelli *et al.*, 2014; Liu *et al.,* 2016).

2.7 Generation of neuronal subtypes relevant for neurologic diseases modeling

The research on neurological diseases has particularly benefited from the advent of hPSCs cultures since their high flexibility to the *in vitro* model such pathologies coupled to the enormous potentialities for drug discovery. Efficient differentiation of PSCs into neurons can be achieved by two different protocols: an embryoid body-based 3D induction method, and a monolayer based 2D induction method (Chambers *et al.,* 2009; Zeng *et al.*, 2010). The first crucial step in the generation of neural precursor cells is by efficiently inducing pluripotent stem cells (PSCs) into early neuroepithelial progenitors (NEP) of the dorsal telencephalon system (Chambers *et al.,* 2009). These NEPs have the potential to maturate into defined region-specific CNS neuronal subtypes using sequential exposure to appropriate *in vitro* environmental signals. The neural induction protocol involves in dissociating PSCs and plating them on a feeder (e.g. astrocytes, mouse embryonic fibroblast) or feeder-free (e.g. Matrigel, Laminin) adherent culture

system. The media for neural induction usually consist of the neurobasal medium or DMEM/F12 medium, or combination of both. The cells are rapidly induced with antagonists, either LDN or noggin to inhibit the BMP pathway and SB431542 to inhibit the TGF-ß pathway, along with additional components (e.g. insulin, ascorbic acid, bFGF) to enhance neuronal precursors (Chambers *et al.,* 2009). The primitive neuroectodermal aggregates (3D system) or neuroepithelial sheets (2D system) are then plated on the adherent substrate to promote the definitive neuroectoderm fate. Upon reaching the "end phase," NPCs are organized into polarized structures called neural rosettes. These neural rosettes are selected and cultured for several passages and then directed towards astroglial progenitors with different combinations of morphogens (CNTF, SHH, FGF, RA) in the defined culture medium. Numerous studies have utilized each of these methods, often with minor variations. However, it is not always clear why a particular method was chosen, and so it is very difficult to evaluate the exact effect of these small changes. In the next paragraphs, we have summarized the most efficient ways to generate specific neuronal subtypes from PSC-derived NPCs through *in vitro* culture. The schematic picture (Figure 6) below describes some of the recent studies providing promising strategies for controlled generation of specific neuronal subtypes useful to model neurological disorders with hPSCs.

Dopaminergic neurons: Dopaminergic (DA) neurons located in the *Substantia Nigra Pars Compacta* play a critical role in regulating postural reflexes and represent the neuronal subtype selectively affected in Parkinson's disease (PD). Neurons with DA identity have been obtained from hESCs after the first step of neuroectodermal induction on stromal feeder cell, a second step in which cells were instructed to acquire the ventral midbrain/hindbrain fate by exposure to fibroblast growth factor 8 (FGF-8) and Sonic hedgehog (SHH), and a terminal DA differentiation in the presence of ascorbic acid, brain-derived neurotrophic factor, glial cell derived neurotrophic factor, TGFß3 and Dibutyryl cAMP (Perrier *et al.*, 2004). More recently, it has been reported the efficient conversion of human embryonic stem cells in DA neurons through a protein-transduction based method, by the direct delivery of a trans-activator of transcription (TAT)-LMX1A recombinant fusion protein, as an alternative to genetic modification (Fathi *et al.*, 2015). Therefore, to produce DA neurons *in vitro*, it is essential to add FGF8 to *in vitro* culture system (Day 9 onwards) to pattern the progenitors at the caudal stage.

Cortical neurons: Cortical neurons, involved in the higher cognitive functions, are drastically affected in several psychiatric disorders, characterized by alteration in differentiation, migration and synaptogenesis processes. The majority of the anterior neuroepithelial (NE) cells become progenitors of the cerebral cortical identity. This ''default'' cerebral cortical identity is partly due to the expression of numerous WNT ligands in the differentiating neuroepithelial. Thus by regulating the concentration of WNT and SHH, the NE cells can be patterned to the most ventral part of the forebrain, i.e., equivalent of producing cortical neurons. Shi *et al*. have exploited hPSCs to generate NEPs specifically instructed to originate cortical projection neurons in a fixed temporal order (Shi *et al.,* 2012). Likewise, Boissart *et al*. reported the generation of late cortical progenitors (LCP) from human hPSC-derived NEPs, which can efficiently generate mature glutamatergic neurons of the superficial cortical layers (Boissart *et al.,* 2013).

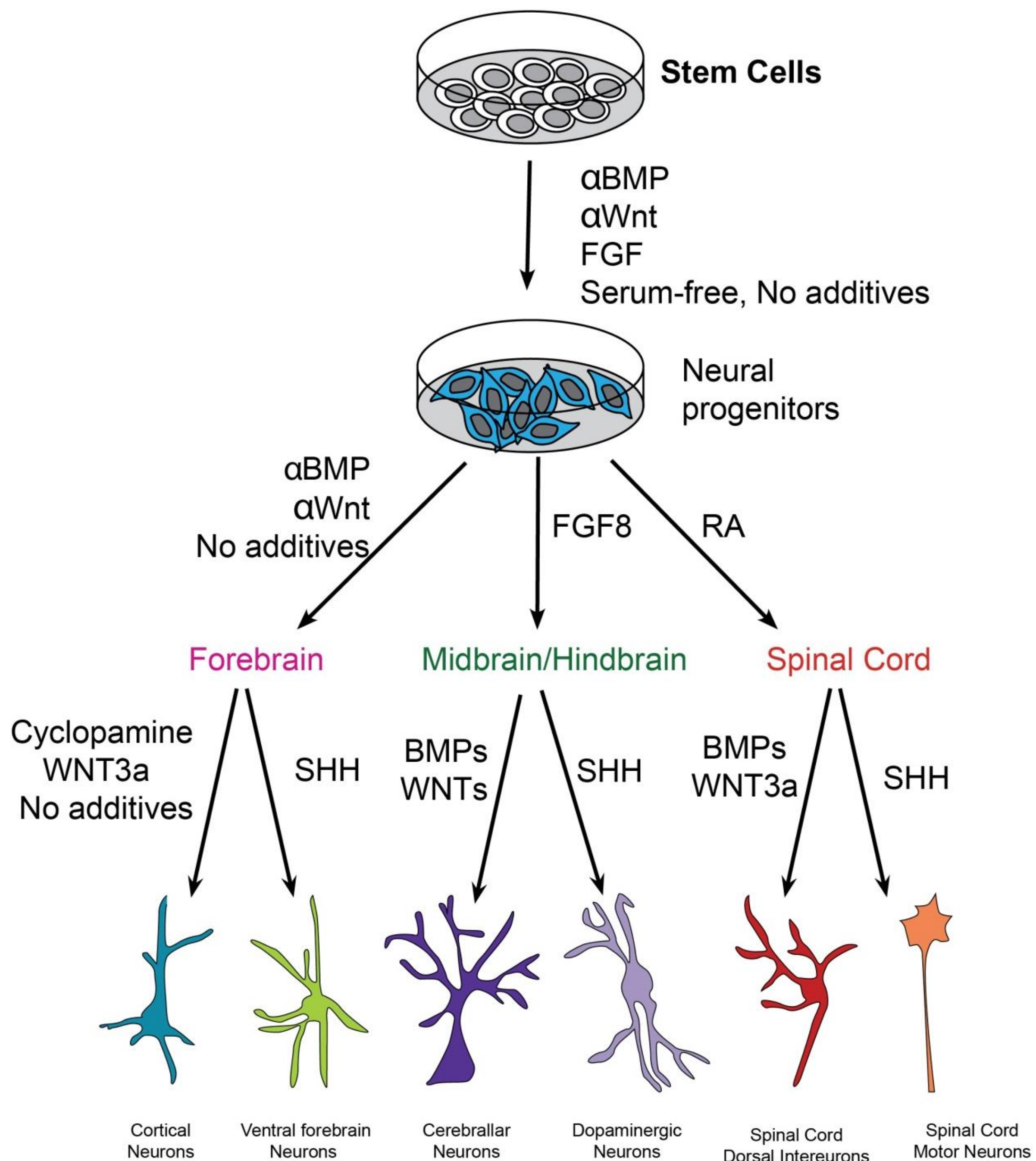

Figure 6: Representation of *in vitro* neuronal subtype-specific differentiation protocols of hPSCs. Dopaminergic (DA) neurons can be originated from NEPs, smNPCs, or pNSCs by exposure to specific stimuli. Cortical neurons can be obtained from NEPs, NEP-derived LCP or three-dimensional aggregates called SFEBq. Motor neurons have been differentiated from hPSC-derived NSC spheres or EBs. Interneurons can be differentiated from hPSC-derived MGE-like progenitors but also directly from NEPs. This figure is adapted from (Corti *et al.*, 2015).

Motor neurons (MN): Retinoic acid and SHH play crucial roles during motor neurons (MNs) development through the rostrocaudal and dorsoventral axis of the neural tube. Wichterle *et al.* generated the first mouse motor neurons protocol from mouse ESCs (Wichterle *et al.*, 2002). Later Eggan's group translated this protocol to human cells. To specify motor neuron progenitors, a combination of SHH and CHIR (Day 0-12) enriches the OLIG2-expressing progenitors, thus producing motor neurons at over 90% purity. If administration differs, it results in varying degree of purity (Du *et al.,* 2015). For instance: early exposure to SHH lead to interneurons (INs) differentiation, whereas a protracted use pushes cells toward oligodendrocytes differentiation *(Jha et al*., 2015).

2.8 Modeling neurodegenerative disease with PSCs derived neuroglia

Great strides have been made in the advancement of hPSC technology and their ability to generate pluripotent-derived cellular models. It is worthwhile to mention the success of iPSC technology in regards to neural-glial disease modeling including Parkinson's disease (Rhee *et al.*, 2011; Sánchez-Danés *et al.*, 2012), demyelination (Tokumoto *et al.*, 2010; Czepiel *et al.*, 2011; Pouya *et al.*, 2011), retinal regeneration (Parameswaran *et al.*, 2010; Tucker *et al.*, 2011), nerve degeneration (Wang *et al.,* 2011) and various others (reviewed by (Saporta, Grskovic and Dimos, 2011)). Recent studies by Chen *et al*. shed light on understanding the disease phenotypes of Down syndrome (DS) using an iPSC tool (Chen *et al.*, 2014). This study tested minocycline (an FDA approved drug) to correct the pathological phenotypes of DS astroglia. Notably, their results demonstrated higher levels of GFAP/S100 beta expression as compared to control astroglia. Therefore iPSCs technologies are now considered to be a valuable platform to model diseases and improve our understanding of pathomechanism. Furthermore, they can be employed for drug screening and testing. In the following section, we would like to discuss some of the diseases that could see, or already have seen benefits from this technology.

2.8.1 Parkinson Disease (PD)

PD is a neurodegenerative age-related disorder characterized by several motor impairments, such as tremor, rigidity, postural instability, and other non-motor symptoms, as psychiatric manifestations, sleep disturbance and hyposmia (Jankovic, 2008). Different groups have reported the derivation of hiPSC lines from familial PD patients with α-synuclein (Devine *et al.*, 2011), Parkin (Imaizumi *et al.*, 2012), LRRK2 (Nguyen *et al.*, 2011), and PINK1 mutation (Seibler *et al.*, 2011). These studies demonstrate that overlapping phenotypes are present in familiar and sporadic neurons from hPSC lines from PD patients, indicating that hiPSC-based *in vitro* models might be useful to capture the patients' genetic complexity.

2.8.2 Amyotrophic lateral sclerosis (ALS)

ALS is an adult-onset neurodegenerative disease manifested by degeneration of motor neurons in the motor cortex, brain stem, and spinal cord, resulting in muscle paralysis and ultimately motor neuronal death (Rowland and Shneider, 2001; Kiernan *et al.*, 2011). In 2014, Zhang colleagues generated iPSCs from individuals with SOD1 mutations to investigated early pathological events in motor neurons derived from these iPSCs. In their study, they showed that SOD1-mutant motor neurons exhibited neurofilament aggregation and altered stoichiometry for the neurofilament subunits (Chen *et al.*, 2014a). In the same year, Eggan *et al*. showed transcriptional changes in motor neurons derived from the iPSCs of individuals with ALS caused by pathogenic repeat expansions in the C9orf72 locus, suggesting that these distinct disease-causing mutations act through common pathways (Kiskinis *et al.*, 2014).

2.8.3 Alzheimer's disease (AD)

AD is characterized by the progressive deterioration of cognitive functions such as memory and mental processing. Most cases of AD are sporadic, but about 1-2% are genetically linked with the early onset (EOAD) of dementia and are associated with mutations in amyloid precursor protein (*APP*), presenilin-1 (*PSEN1*) or presenilin-2 (*PSEN2*). There are two major

histopathological hallmarks in the brain of AD patients, the deposition of extracellular senile plaques (SPs) composed of the amyloid-beta (Aβ) peptide, and neurofibrillary tangles, which are intracellular inclusions of hyperphosphorylated TAU protein in selective regions of the brain (Sidoryk-Wegrzynowicz *et al.*, 2011). SPs are deposits of extracellular Aβ protein derived from Aβ42, a peptide fragment of 42 amino acid residues derived from the sequential step of proteolytic processing of amyloid precursor protein by β and γ secretase. As the disease progresses, synaptic loss and neuronal death become prominent, which consequently lead to the shrinkage of the brain.

Highlights on recent progress of modeling AD with iPSCs are provided below. This section was adapted from our submitted unpublished paper. Yagi *et al*. established the first proof-of-principle of iPSC-based modeling where they generated iPSCs from familial Alzheimer's disease (fAD) patients carrying mutations in the AD causative genes, PSEN1 (A264E) and PSEN2 (N141I). The supernatant of neuronal cultures from the AD lines exhibited an elevated ratio of extracellular Aβ42 to Aβ40 compared to controls (Yagi *et al.*, 2012), which was observed in AD patients (Iwatsubo *et al.,* 1994). Conversely, the treatment of a γ-secretase inhibitor significantly lowered the Aβ levels and the Aβ42/Aβ40 ratio (Yagi *et al.,* 2012). Sproul *et al.* developed additional patient- iPSC lines with mutations in PSEN1 (A264E and M146L) and found similar Aβ42/Aβ40 ratios in the neural progenitor cells derived from AD iPSCs compared to the controls (Sproul *et al.*, 2014). In 2012, Israel *et al.* generated iPSCs from two fAD patients carrying APP duplication (APP^{Dp}) and two sporadic Alzheimer's disease (sAD) patients (Israel *et al.*, 2012). The authors, however, detected higher levels of Aβ40 secretion instead of Aβ42 in neuronal cultures derived from their AD lines. They speculated that their inability to measure Aβ42 was due to low Aβ42 abundance caused by the small number of neurons in their cultures. Tauopathy, which is another pathological hallmark of AD, was also explored in a study where neurons derived from APP^{Dp} and sAD iPSC exhibited an abnormal accumulation of phosphorylated tau (phospho-tau) and activation of tau kinase GSK-3β (aGSK-3β). Treatment of β-secretase, which enzymatically cleaves APP before γ-secretase, reduced Aβ40 levels in one of the APP^{Dp} and sAD line as well as lowering the level of phospho-tau and aGSK-3β. The β-secretase is localized to endosomes, and the authors identified the abnormal presence of large endosome (RAB5+) in their patient-derived neurons, which is consistent with the observation in post-mortem brains of AD patients. Increased APP cleavage products, APPsβ and Aβ, were detected in the AD neurons directed to a forebrain fate, suggesting this mutation affects the APP processing by both β- and γ-secretases (Muratore *et al.*, 2014). Elevated levels of extracellular Aβ42/Aβ40 ratio, total tau, and phospho-tau were also detected in the AD cells compared to controls (Muratore *et al.*, 2014). The treatment of cells with Aβ antibodies reversed the total tau level, and thus provides further evidence for an Aβ-tau connection. In 2013, Kondo *et al.* generated iPSCs from two fAD patients carrying a specific mutation in APP (E693Δ, V717L) and two sAD patients (Kondo *et al.*, 2013). Intracellular accumulation of Aβ oligomers and reactive oxygen species (ROS) were detected in neurons as well as astrocytes derived from one of the fAD and sAD lines (Kondo *et al.*, 2013). The treatment of β-secretase inhibitor alleviated these cellular abnormalities, supporting the role of Aβ as a stressor to neurons and glial cells (Kondo *et al.*, 2013).

2.9 The Role of Astrocytes in the CNS

Astrocytes play a direct and critical role in the developing CNS in maintaining an optimal environment for the normal development and function of neurons. Some examples of astrocytic functions include energy supply, the formation of the blood-brain barrier (BBB), and removal of toxins and debris (described below). Impairments in these functions, as well as physiological fluctuation in glutamate/K^+ levels, can trigger or exacerbate neuronal dysfunction (Zhang *et al.,* 2016). Based on their important and physiological role, it is not at all surprising that changes in astrocytes can directly affect the behavior of rodents (Franke and Kittner, 2001).

Energy supplies for neurons. One of the oldest known functions of astrocytes is to supply energy in the form of lactate to neurons. Glucose is mainly stored as glycogen in astrocytes, where it is metabolized to pyruvate and lactate and then transported via monocarboxylate transporters (MCTs) across the cell membrane. The transported lactate is then utilized by neighboring neurons and metabolized (Magistretti *et al.*, 1999).

Maintenance of the cellular homeostasis of the brain. Apart from energy supply, astrocytes are involved in maintenance of brain homeostasis through multiple dynamic equilibrium adjustments, including water balance, ion distribution, glutamate buffering and recycling (Wang and Qin, 2010; Coulter and Eid, 2012).

Control of cerebral glutamate levels. Besides astrocytes can control cerebral glutamate levels (Stobart and Anderson, 2013). Glutamate that is taken up by the astrocytes is converted to glutamine by glutamine synthase (GS), then later passed back to the synaptic terminal where it is converted back to glutamate (Danbolt, 2001; Parpura and Verkhratsky, 2012). There is increasing evidence that the uptake of glutamate also induces glycolysis in astrocytes, resulting in the production and secretion of lactate for the neighboring neurons (Stobart and Anderson, 2013; Bélanger and Magistretti, 2009; Ricci *et al.*, 2009). This mechanism, the astrocyte to neuron lactate shuttle, regulates lactate delivery in an activity-dependent manner (Pellerin *et al.*, 1998; Stobart and Anderson, 2013).

Formation and maintenance of the blood-brain barrier. Together with endothelial cells and pericytes of the brain micro vessels, astrocytes form the blood-brain barrier (BBB), a physical diffusion barrier which restricts the exchange of most molecules between blood and brain (Abbott *et al.,* 2006; Macvicar and Newman, 2015). Astrocytes are also involved in regulating cerebral blood flow by a K^+ siphoning mechanism, releasing K^+ onto blood vessels from their end-feet in response to neuronal activity (Paulson and Newman, 1987). It has been suggested that the release of prostaglandins from astrocytes results in increased Ca^{2+} that evokes vessel dilation (Zonta *et al.*, 2003). Likewise, they are also involved in regulating BBB permeability from the bloodstream to brain parenchyma by the activation of tight junction proteins through NF-κB (Brown *et al.*, 2003; Abbott *et al.,* 2006). BBB defects are involved in many neuroinflammatory and neurodegenerative diseases, including multiple sclerosis, where the specialized brain endothelial cells which comprise the BBB are diminished, causing a loss of protective function during the progressive phase of the disease (Weiss *et al.*, 2009).

2.10 Role of astrocytes in *in vitro* neuronal maintenance and maturation

There is now abundant evidence to support the notion that astrocytes are actively involved in the formation and refinement of neural networks (Oberheim *et al.*, 2006; Araque and Navarrete,

2010). During development, billions of neurons connect to form functional networks via synapses, with the control of synapse development by astrocytes highly conserved across species. A distinctive attribute of astrocytes in synapse formation is to increase the number of synaptic structures (dendritic spine) within the neural circuits (Ullian *et al.*, 2001; Slezak and Pfrieger, 2003; Stevens *et al.*, 2007; Stipursky *et al.*, 2011; Clarke and Barres, 2013). The first evidence for astrocytes being involved in synapse formation came from the rodent retinal ganglion cells study, which showed that culture with astrocytes resulted in a tenfold increase in the excitatory synapse and synaptic functionality (Meyer-Franke *et al.*, 1995). Later, *in vitro* studies confirmed that astrocytes could also instruct synapse formation for human neurons (Diniz *et al.*, 2012).

Astrocytes are also involved in the refinement of the neural network by synaptic pruning the elimination of extra synapses to increase the precision and efficiency of neural circuits (Clarke and Barres, 2013). The mouse retinogeniculate system, an excellent model system for studying synapse refinement and elimination (Hong and Chen, 2011), has been used to show that signals released from astrocytes in the postnatal brain induced the expression of the complement component C1q that executes synapse elimination by astrocytes via phagocytosis (Stevens *et al.*, 2007). Notably, astrocytes employ this mechanism throughout the nervous system (e.g. in the uninjured brain or response to glioma or trauma). However, further work is required to investigate the phagocytic pathway of astrocytes in human models.

2.11 Generation and differentiation of astroglial cells *in vitro*

During organismal development, the fate of the respective cell types is determined by the exact timing and concentration of growth factor/patterning signals at given locations. With knowledge of the patterning signals, *in vitro* astrocytogenesis of defined subpopulations could be achieved by exposing human PSC-derived primitive neuroepithelia to a set of diffusible signaling molecules, directing their differentiation into subpopulations that would arise *in vivo* in discrete regions along the neural tube. This process could generate functionally diversified classes of glial cells. A similar approach is commonly used for neurons (Kirkeby *et al.*, 2012). For instance, FGF and retinoic acid determine rostrocaudal identity, whereas WNTs, BMPs, and SHH are required to specify NPCs along the dorsoventral axis. We know that astroglial progenitors generated in the absence of mitogens carry a dorsal-anterior identity by expressing Otx2 but not Hoxb4 or Nkx2.1, while astroglial progenitors generated in the presence of retinoic acid express Hoxb4 but not Otx2 (Liu and Zhang, 2011).

2.12 Importance of Astrocytes in neurological diseases

Since the importance of astrocytes for functional neuronal networks has long been underestimated, it is not surprising that their central role in many neurological disorders was equally neglected. Experiments on mouse models of human neurological diseases including diverse neurodegenerative diseases (e.g. Alzheimer's disease, Amyotrophic lateral sclerosis, Parkinson's disease, and spinocerebellar ataxia) and neurodevelopmental disorders (e.g. Alexander's disease, Autism spectrum disorders, Epilepsy and Rett syndrome) have led to advances in understanding astrocyte biology.

Alexander disease is a progressive astrogliopathy caused by a dominant gain-of-function mutation in the glial fibrillary acid protein (GFAP) gene those maps to chromosome 17q21

(Brenner *et al.*, 2009). It is a primary disease of astrocytes that affects neural development and causes mental retardation, seizures, and megaencephaly in early childhood (Prust *et al.*, 2011). In the case of **Hepatic Encephalopathy** patients, neurons appear to be morphologically normal, but astrocytes show signs of Alzheimer type II degeneration, i.e. nuclear enlargement, prominent nuclei, chromatin changes and neurotransmitter receptor alteration. (Butterworth, 2010; Felipo and Butterworth, 2002). There have been some animal studies evaluating BBB integrity in acute liver failure, but there has been less research on this subject conducted on humans. To our knowledge, Chen *et al.* evaluated the hepatoprotective property of 3-genes iPSC transplantation in a carbon tetrachloride (CCl_4)-induced AHF model in mice (Chen *et al.*, 2012). **Neuromyelitis Optica (NMO)** is a primary astrocytopathy disease affecting the CNS. NMO was first described in the 19th century and was long considered a variant of multiple sclerosis (Marignier et al. 2010). The disease is commonly associated with diffuse cerebral white matter lesions that resemble acute disseminated encephalomyelitis, and severe demyelination affecting the optic nerve and spinal cord. The evidence of this disease reveals the loss of neurons and astrocytic damage (Wingerchuk *et al.*, 2007).

3. MATERIALS AND METHODS

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

3.1 Mouse embryonic stem cell culture

The mouse embryonic stem cell (mESC) line HM1 (129/Ola mouse strain origin, described by Selfridge et al., 1992; was kindly provided by Roslin Institute, UK; at passage 19.) was used in the experiments. The pluripotent cells were maintained in two different ways either using (i) CGR8 medium: Glasgow modified Eagle's medium (GMEM) containing 10% (vol/vol) fetal bovine serum, 2mM sodium pyruvate, 2 mM glutamax, 100µM nonessential amino acids (NEAA), 50µM β-mercaptoethanol (β-ME), 50U penicillin/mL, 50µg streptomycin/mL, and 1000U/mL mouse LIF (ii) or in Dulbecco's modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12) containing 3mg/mL of d-(+)-glucose containing 10% (vol/vol) ESC grade-fetal bovine serum, 2mM sodium pyruvate, 2 mM glutamax, 100µM nonessential amino acids (NEAA), 50µM β-mercaptoethanol (β-ME), 50U penicillin/mL, 50µg streptomycin/mL, and 1000U/mL mouse LIF. The cells were passaged before reaching 70% confluency (approximately every 2 Days). The protocol involves the use of early-passage mitotically inactivated (Mitomycin C treated) mouse embryonic fibroblast feeders (MEF) for maintenance of mESCs *in vitro*. Before 2D neural induction using mESCs, the cells were transferred to feeder free condition where ESCs were cultured on gelatin-coated dishes in the presence of 2i inhibitors to maintain the pluripotency (Ying, Wray, *et al.*, 2008). Inhibitors were used at the following concentrations unless otherwise specified: 1 µM of CHIR99021-GSK inhibitor and 0.8 µM of PD184352-MERK (Ying *et al.*, 2008; Nichols and Smith, 2009).

3.2 Induction of neuronal differentiation of mouse ESCs

3.2.1 2D Monolayer induction

Mouse pluripotent cells were induced to differentiate into the neuronal lineage as previously described, with some modifications (Kleiderman *et al.*, 2016). Mouse ESCs were harvested into single cells using 0.05% (wt/vol) Trypsin, then seeded at a density of 5.6×10^5 cells/mL in differentiation medium (N2B27 medium) onto 10 cm bacteriological dishes precoated with 0.1% gelatin. Pluripotent cells were allowed to uniform monolayer for 6 Days. The medium was changed every other day. Upon the end of induction, the cells were treated with accutase for 3 min, resuspended with N2B27-medium and filtered through a 70-mm cell strainer into 50 mL polypropylene tubes to form single cells. After centrifugation at 1000rpm for 3 min, all cells were plated onto Poly-ornithine, and Laminin (POL/L; 0.002%/1µg/cm^2) coated T75 Nunclon flasks in N2B27-medium supplemented with (10 ng/mL) FGF2 and (10 ng/mL) EGF. The cells were harvested in POL/L dishes and were cryopreserved in FBS containing 10% DMSO at 1x10^6 cells/mL. To promote astrocyte differentiation 2D, induced NPCs were used along with cytokine to induce astroglial differentiation.

3.2.2 Astrocyte differentiation of mouse ESCs

NSC cultures from passage 8 or later at 80% confluency were treated with 0.05% trypsin for 15 seconds, re-suspended in N2B27-medium, and filtered through a 70 µm cell strainer prefilled with PBS, centrifuged (500g, 3 min), and re-suspended in the medium. Cells were plated at a density of 30.000 cells/cm^2. Nunclon Delta dishes/plates were coated with 10 µg/ml poly-L-ornithine-hydrobromide in PBS for 2 hours at 37°C, washed twice with PBS, and coated with 2 µg/ml Laminin in PBS overnight. Laminin was aspirated, and the cell suspension was added to dishes/plates. **For mouse astrocyte differentiation:** For astrocyte differentiation, late NPC passage was used. The reason for using later NPC passage is that the cells in these initial cultures have different morphologies and cell-cycling behaviors are different, for instance: some of them growing faster than others do. This method was adapted from (Kleiderman *et al.*, 2016). Medium was changed every other Day from Day 1, and experiments were performed at Day 5 of differentiation, if not otherwise stated. If cells were cultured for longer periods, the medium was changed every other day using N2B27-medium, containing 20 ng/ml BMP4 (Kleiderman *et al.*, 2016).

3.2.3 Primary rat astrocyte culture

Primary glial cell cultures are the most commonly used *in vitro* model for neurobiological studies. Cryopreserved Rat primary cortical astrocytes were purchased from Thermo Fisher Ltd. The astrocytes were isolated from cortices of Sprague-Dawley rats at embryonic Day 19 (E19) and cryopreserved at the end of the first passage. The cells were thawed in astrocyte growth medium (containing 85% DMEM /F12-high glucose and 15% FBS). The rat astrocytes were maintained in uncoated; tissue culture treated flasks for maintenance and expansion. Upon reaching 100% confluence (4-5 Days intervals), the cells were passaged for expansion, using Accutase (3 minutes, RT) and were seeded at a seeding density of 2×10^4 cells/cm^2. For differentiation experiments, the rat astrocytes were cultured on coverslips for 4 days.

3.3 Human pluripotent stem cell culture

Human iPSCs used in this study were generated by Sendai virus-based reprogramming as described earlier (Nemes *et al.*, 2016; Chandrasekaran *et al.*, 2016; Ochalek *et al.*, 2016). Five hiPSC lines were compared: two healthy individuals derived control lines (CTL-1 and CTL-2, published in (Zhou *et al.*, 2016), and three Alzheimer's disease lines (DL-1, DL-2, and DL-3). The hiPSC line DL-1 was originated from an early familial AD (eFAD) patient with known pathogenic mutation in PSEN1 gene (Nemes *et al.*, 2016) while the other two lines were reprogrammed from late-onset sporadic cases without known genetic background (DL-2, (Chandrasekaran *et al.*, 2016); and DL-3 (Ochalek *et al.*, 2016). Phase contrast images of human iPSCs and the characterization of DL-2 line is shown in (Figure 9B). All other iPSC were characterized similar to DL-2 as published. All the clones were maintained on Matrigel (BD Matrigel; Stem Cell Technologies) in mTESR1 (Stem Cell Technologies) culture media. Cultures were fed daily with mTESR1 and passaged every 5-7 Days for colony growth, following the instructions of the manufacturer.

3.4 Induction of neuronal differentiation of human iPSCs

3.4.1 Monolayer based (2D) neural induction

Dual inhibition of the SMAD signaling pathway was chosen as the 2D neural induction method (see details in Supplementary Figure 1). The hiPSCs were directed towards neural fate by the administration of 10 µM SB431542 and 500 ng/mL Noggin (R&D) (Chambers *et al.*, 2009; Shi *et al.,* 2012) in neural induction medium (NIM) (DMEM/F12: Neural basal medium, supplemented with 1xN2 and 2xB27, 2 mM glutamine, 1x non-essential amino acid, 100 µM ß-mercaptoethanol, 5 µg/mL insulin, 5 ng/mL bFGF) until Day 10. Tissue culture plates were coated with Poly-L-ornithine and Laminin (POL/L; 0.002%/1 µg/cm^2), (Roche). By Day 10, neural epithelial sheets had developed several neural rosettes, which were manually picked under a microscope in sterile laminar flow and re-plated onto POL/L plates. At this time point, we analyzed cells for NEP. Up to passage 4, rosette-like structures were plated *en bloc* on POL/L plates, without dissociation. From passage 4, the cells were passaged using accutase, and the NPCs were seeded as single cells (min 50.000 cells/cm^2) for further expansion in neural maintenance medium (NMM), (DMEM-F12:Neural basal medium, supplemented with 1xN2 and 2xB27, 2 mM glutamine, 1x non-essential amino acid, 25 µM ß-mercaptoethanol, bFGF (10 ng/mL) and EGF (10 ng/mL) and maintained on plates coated with POL/L (0.002%/1 µg/cm^2). At this time point, we analyzed cells for progenitors. The efficiency of the neural induction was monitored by flow cytometry (NESTIN, PAX6), immunocytochemical analyses and qRT-PCR for the following markers: *NESTIN, PAX6, SOX1* and *SOX9* (Zhang *et al.*, 2001; Reubinoff *et al.*, 2001; Gerrard, Rodgers and Cui, 2005). NPC cultures with at least 70% of cells positive for PAX6 and NESTIN were considered as a successful induction (Chambers *et al.*, 2009; Shi *et al.,* 2012). For terminal differentiation into cortical neurons, the cells were plated on POL/L (0.002%/2 µg/cm^2) at a seeding density of 40.000 cells/cm^2 with NMM medium. The medium was changed every 3-4 Days during the terminal differentiation. The efficiency of terminal differentiation was monitored by immunocytochemical staining and qRT-PCR for Tubulin Beta 3 Class III (TUBB3) and MAP2 expression at week 5. In the current study, iPSCs derived NPCs from passage 5 up to passage 6 were differentiated for 8 weeks for the patch clamp studies and 0.7 weeks (5 Days) for neurite length measurements.

3.4.2 Sphere based 3D neural induction

To generate 3D spheres, the iPSCs cells were dissociated using gentle cell dissociation buffer and were plated on non-adherent plates to enhance the cell aggregation. At the time of plating (Day 1), the cells were seeded as clumps with an average clump size of 80-100 cells/clump and the cell concentration was approximately 0.5-1.0x10^7 cells/mL in NIM. The NIM media, containing Noggin (500 ng/mL) and SB431542 (10 µM), was changed after 24 hours, and then every other Day until Day 9. Within the cell aggregates, rosette-like structures could be observed (Bez et al. 2003) under phase contrast. At this time point, we analyzed cells for NEP. On Day 8, the floating spheres were moved onto POL/L (0.002%/1 µg/cm^2) coated plates for attachment and outgrowth. By the end of Day 13, the attached spheres formed neuronal rosettes and were clearly identifiable (Zhang *et al.*, 2001; Emdad *et al.*, 2012). To detach the neural rosettes, they were gently flushed from the plate surface by treating with accutase for 3 minutes and were plated as small clumps in POL/L coated dish in NMM media, supplemented with 10 ng/mL of

bFGF and 10 ng/mL of EGF. Upon reaching confluence, NPCs were passaged and plated onto new POL/L plates, expanded and analyzed in a similar way as in the case of the 2D neuronal induction method, in NMM media supplemented with 10 ng/mL of bFGF and 10 ng/mL of EGF. NPCs were characterized with ICC, FACS, and qRT-PCR, frozen or used in further applications (Supplementary Figure 1).

For terminal differentiation, the 3D neural induction was differentiated identically to the 2D neural induction method. The cells were plated onto POL/L-treated plates (0.002%/2 μg/cm^2) at a seeding density of 40.000 cells/cm^2 in NMM medium. The medium was changed every 3-4 days over the course of the terminal differentiation. The efficiency of neural induction and terminal differentiation was monitored as mentioned above in the 2D neural induction section. All samples were collected at the same time point as mentioned in 2D neural induction section.

3.4.3 Astrocyte differentiation of human PSCs

The iPSCs were induced to forebrain derivates to acquire cortical progenitor identity as described above (section3.4). The monolayer derived NPCs were propagated in NMM media for NPC generation. Astrocyte populations (NPCs over p9) were obtained by differentiating the late phase NPCs on POL/L coated plates or tissue culture flasks.
NPC cultures from passage 8 or later at 80% confluency were treated with 0.05% trypsin for 15 seconds, re-suspended in N2B27-medium, and filtered through a 70 μm cell strainer prefilled with PBS, centrifuged (500g, 3 min), and resuspended in the medium. Cells were plated in N2B27-medium, supplemented with 20 ng/ml ciliary neurotrophic growth factor. NunclonTM Delta dishes/plates were coated with 10 μg/ml poly-L-ornithine in PBS for 2 h at 37°C, washed twice with PBS, and coated with 2 μg/ml laminin in PBS overnight. Laminin was aspirated, and the cell suspension was added to dishes/plates. The medium was changed every other day from Day 1, and experiments were performed at Day 35 of differentiation, if not otherwise stated. When cells were cultured for longer periods, the medium was changed every other day using N2B27-medium, containing 20 ng/ml CNTF.

3.5 Fluorescence-activated cell sorting (FACS)

NPCs were collected using 0.5% trypsin to get single cell suspension and were fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature (RT). After fixation, the cells were washed in cold PBS and centrifuged at 200g for 5 minutes. Cells were permeabilized with 0.2% Triton X-100 for 5 minutes at RT; followed by blocking with 1% BSA for 15 minutes at RT. The cells were then incubated with the corresponding antibodies Alexa Fluor-647 mouse anti-NESTIN (BD Biosciences) PE-mouse anti-human PAX6 (BD Biosciences), PerCP-Cy5.5 mouse anti-human SOX1 (BD Pharmingen) or goat anti-human SOX9 (R&D) and for 1 hour at RT, while for the unconjugated primary antibodies isotype specific secondary antibodies were used accordingly (for details see Supplementary Table 1). Cells were washed and analyzed using 'Flow Cytometer Cytomics FC 500' (Beckman Coulter). A red solid state laser 635 nm and an argon laser 488 nm were used to detect NESTIN and PAX6 or SOX1 and SOX9 positive cells. Proper gating and compensation were performed using appropriate controls. Data was analyzed using FlowJo software (version 7.6.5; FlowJo, LLC).

3.6 Immunostaining

Cell cultures were fixed in 4% PFA for 20 minutes at RT and washed 3 times with PBS before pre-incubation with permeabilization solution (PBS plus 0.2% tritonX-100) for 20 minutes. The cells were then blocked for 40 minutes at RT in blocking solution (3% BSA in permeabilization solution). The cells were then incubated with primary antibodies (see details and dilutions in Supplementary Table 1) overnight at 4°C. On the next day; cells were washed with PBS and isotype specific secondary antibodies (for details see Supplementary Table 1) were diluted in blocking buffer and applied for 1 hour at RT. The cells were washed 3 times with PBS and mounted with Vectashield mounting medium containing DAPI (1.5 µg/ml; Vector Laboratories), which labeled the nuclei of the cells. Negative controls for the secondary antibodies were performed by omitting the primary antibodies. Samples were visualized on a fluorescence microscope equipped with a 3D imaging module (AxioImager system with ApoTome, Carl Zeiss MicroImaging GmbH) controlled by AxioVision 4.8.1 software.

Fixed 3D neural induction derived spheres were embedded in Shandon Cryomatrix gel (Thermo Fischer Scientific) according to the manufacturer's instructions, and 10 µm parallel sections were cryosectioned (Leica CM 1850 Cryostat, Leica GmbH) and stored at -20°C freezer until use. Immunostainings was performed as above, and sections were analyzed using FluoView FV10i confocal laser scanning microscope (Olympus Ltd, Tokyo, Japan). Due to the specificity of this process, Tamás Bellák (BioTalentum Ltd.) did the cryosectioning, immunostaining of cryosectioned samples and confocal imaging. The protocol and results are presented here with his permission.

3.7 Electron microscopy

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

Evaluation of the ultrastructural characteristics of the 2D and 3D neural induction derived NPCs was performed in one control (CTL-1) line to identify whether any morphological differences could be observed in the organelles between the 2D and 3D neural induction methods. The 2D neuroepithelial sheets grown on POL/L coated coverslips (at Day 8 of the induction phase) and the 3D neural induction derived free-floating spheres (at Day 8 of the induction phase) were fixed with a fixative solution containing 3.2% PFA, 0.2% glutaraldehyde, 1% sucrose, 40 mM $CaCl_2$ in 0.1 M cacodylate buffer for 24 hours on 4°C. Samples were rinsed for 2 Days in cacodylate buffer, then postfixed in 1% ferrocyanide-reduced osmium tetroxide (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), 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 Olympus Morada 11 megapixel camera and iTEM software (Olympus).

3.8 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from NPCs and differentiated neurons using the RNeasy mini extraction kit (Qiagen) according to the manufacturer's protocol. 500 ng of RNA was transcribed using Superscript III VILO cDNA synthesis kit (Thermo Fisher Scientific). The PCR conditions were subjected to 94 °C, 3 min, initial denaturation; followed by 40 cycles of 95 °C, 5 seconds, denaturation; 60 °C 15 seconds, annealing and 72 °C 30 seconds, elongation. The amplification reactions were carried out in a total volume of 15 µL using SYBR Green Master Mix (Thermo Fisher Scientific). Human *GAPDH* and Beta-2-Microglobulin (*B2M*) were used as reference genes. The data was analyzed using REST software (2009 V2.0.13), and the statistics were analyzed using Graph Pad Prism 5. Values are expressed as ± SEM as indicated by Figure legend text. Statistical significance was tested by unpaired student t-test (two-tailed) for differences between two groups, and by one-way ANOVA with a Tukey's post-test for testing differences between two or more groups. Statistically significant differences were determined when p values were less than 0.05 ($p<0.05$). Oligonucleotide primers used in this study are listed in Supplementary Table 2. In the current study, iPSC-derived NPCs (from 2D & 3D) were differentiated towards neurons for 5 weeks (35 Days) for the qRT-PCR analysis. Statistically significant differences were determined by p values less than 0.05 ($p<0.05$). Probes used in this study are listed in Supplementary Table 2.

3.9 Neurite length measurements

Human iPSC-derived neurons derived from both 2D and 3D neural inductions (at Day 25) were dissociated with accutase for 3 minutes and replated on POL/L (0.002%/1 µg/cm^2) coated coverslips in 24-well plates for neurite length analysis (10.000 cells/cm^2). The plated cells were cultured further for 5 Days and thereafter fixed with 4% PFA. Nuclei were stained with DAPI and the neural cells with anti-β-tubulin III (Covance) and Alexa Fluor 488–conjugated secondary antibody. Images for the blue and green channels (DAPI and Alexa Fluor 488) were taken with a fluorescence microscope equipped with a 3D imaging module (AxioImager system with ApoTome, Carl Zeiss MicroImaging GmbH) controlled by AxioVision 4.8.1 software at BioTalentum Ltd. Neurite length was assessed in three steps using neurite tracer software available from ImageJ (NeuriteTracer is licensed under a Creative Commons Attribution-Non-commercial-ShareAlike 4.0 International License). First, the co-localization plug-ins were used to identify co-localization of soma. Second, the particle analysis function was used to restrict size 50nm^2-infinity. Third, dendrites were traced using the neurite tracer plug-ins (Fournier lab). The mask generated by the particle analysis was then overlaid onto the trace generated by the Neurite Tracer and spines were counted. The analysis was performed as previously described by Pool (Pool *et al.*, 2008)..

3.10 Electrophysiological recordings

The electrophysiology measurements were performed in Opto-Neuropharmacology Group, MTA-ELTE NAP-B, Budapest, By Dr. Árpád Mike and Krisztina Pesti. The protocol and results presented here by their permission.

Standard patch clamp electrophysiology experiments were performed on eight weeks old terminally differentiated neuron cultures. Whole cell recordings were carried out using an Axopatch 200 B amplifier and the pClamp software (Molecular Devices, Sunnyvale, CA).

Currents were digitized at 20 kHz (Digidata 1322A, Molecular Devices) and filtered at 10 kHz. The expression of three different types of ligand-gated ion channels (LGICs) were tested: AMPA receptors using 100 μM kainate, GABAA receptors using 10 μM GABA, and alpha7 subtype of nicotinic acetylcholine receptors (α7 nAChRs) using 10 mM Choline + 10 μM of the positive modulator PNU 120596 [1-(5-Chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea; Tocris Bioscience]. LGIC mediated currents were recorded at -70 holding potential. Voltage-gated ion channel-mediated currents were evoked by depolarizing pulses from a holding potential of -120 mV. Our predominant focus was the voltage-gated sodium channel because expression of sodium channels indicates neuronal phenotype. Activation of sodium and potassium currents was studied using 10 ms voltage steps between -130 and +50 mV in 10 mV increments (Figure 23A, inset). Steady-state availability of sodium channels was studied using a protocol that contained 400 ms long pre-pulse voltage steps from -150 to -20 mV followed by a 10 ms long depolarizing test pulse to 0 mV (Figure 23B, inset). Borosilicate glass pipettes (World Precision Instruments) were pulled with a P-87 micropipette puller (Sutter Instruments) and filled with pipette solution (50 mM CsCl, 60 mM CsF, 10 mM NaCl, 10 mM HEPES, and 20 mM EGTA, pH 7.2). Pipette resistances ranged from 2 to 4 MΩ. Experiments were carried out at room temperature (~25°C). Cells, grown on POL/L treated (0.002%/3 $\mu g/cm^2$) 35 mm Petri-dishes, were transferred to the recording chamber. Culture medium was exchanged to HEPES containing extracellular solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES-Na, 10 mM D-glucose, pH adjusted to 7.3). The osmolarity values (~320 mOsm) of the solutions were balanced with D-glucose. During the experiment, control extracellular solution was perfused continuously (flow rate ~1.66 ml/min). For rapid drug application, we used a pressure-controlled dual U-tube system (K.Szasz *et al.*, 2007). Solution exchange times were in the range of 10–40 ms. In the current study, iPSC-derived NPCs (from 2D & 3D) were differentiated towards neurons for 8 weeks for the patch clamp studies.

3.11 Statistics

For the experiments performed, three independent experiments were carried out for all data presented. Data are reported as the mean ± S.E.M (standard error of the mean). Distribution of data was tested using one-way analysis of variance (ANOVA) with a Tukey posthoc multiple comparison tests and Student's t-test to compare the difference between groups. Statistically significant differences were determined by p values less than 0.05 ($p<0.05$). Data was analyzed and plotted using GraphPad software (Version 5.0). The statical values are illustrated in Supplementary Table 3.

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4. RESULTS

4.1 Differentiation of mouse ECSs towards neuroectodermal lineage

It is long known that cultured mouse embryonic stem cells (mESCs) can generate neurons and other cell types under defined culture conditions (Shiraki *et al.*, 2009). To investigate the ability of neuronal differentiation of mouse ESCs, these pluripotent cells were induced to differentiate into NPCs and neurons through monolayer induction method. The schematic representation of monolayer induction is detailed in Figure 7. When plating the cells on PO/L dishes for terminal differentiation (Day5onwards of differentiation), the cells exhibited a neuron-like appearance with neurite processes organized in a network.

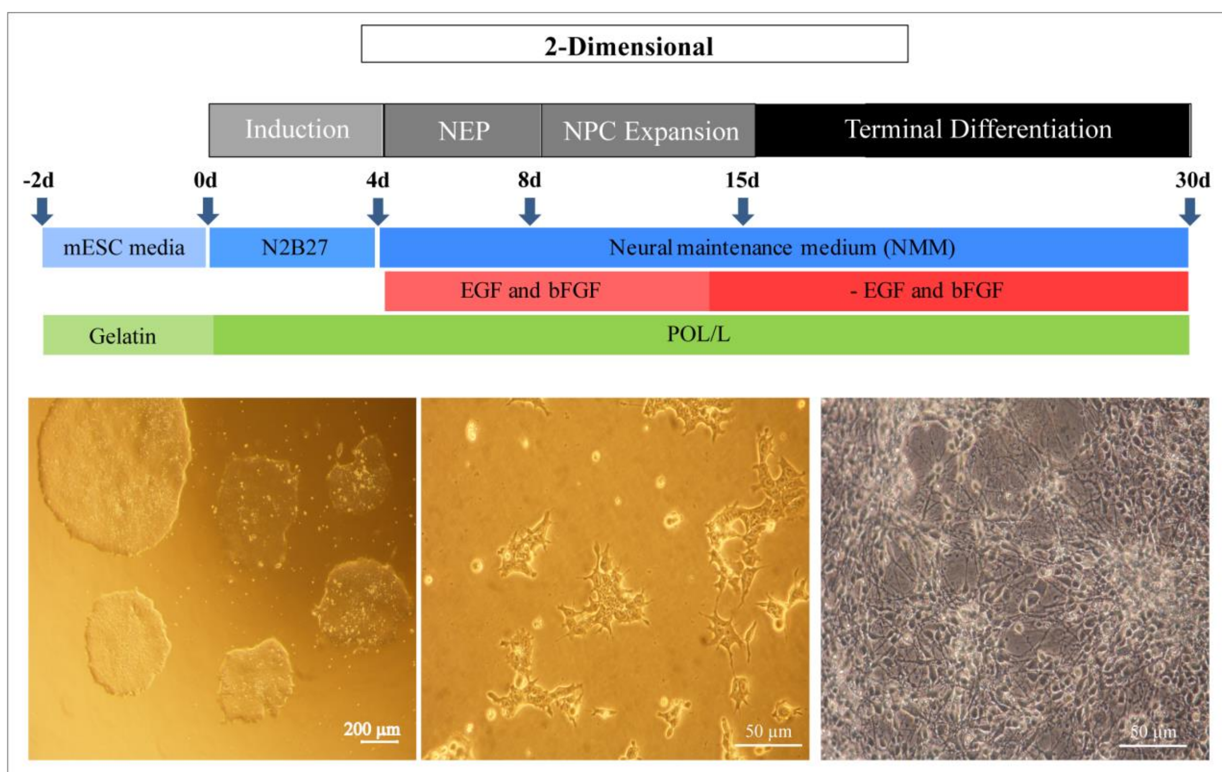
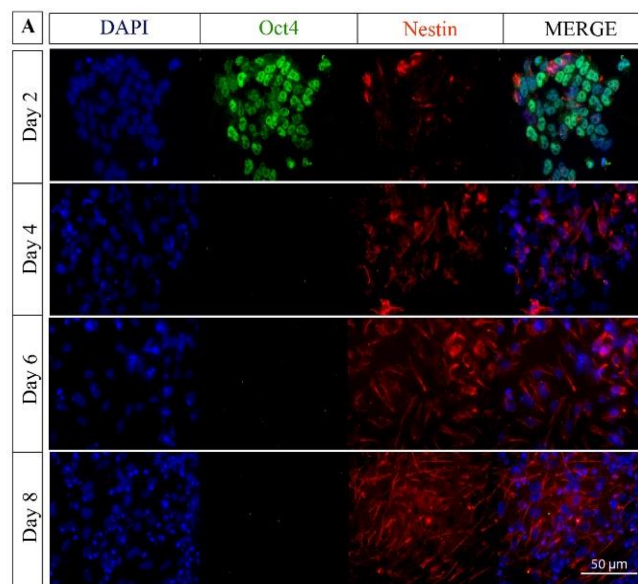


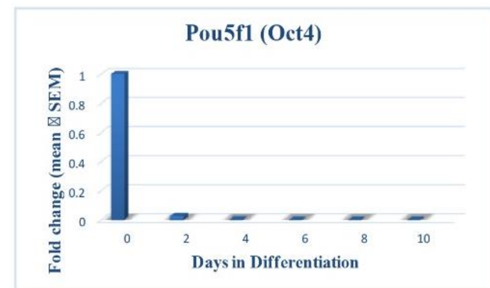
Figure 7. Mouse Neuronal Induction (2D) timeline along with the phase contrast images recorded at mESC stage; Day5 of terminal differentiation; Day 21 of terminal differentiation. These phase contrast images illustrates the morphological change that occurs within the mouse embryonic stem cells during neural induction.

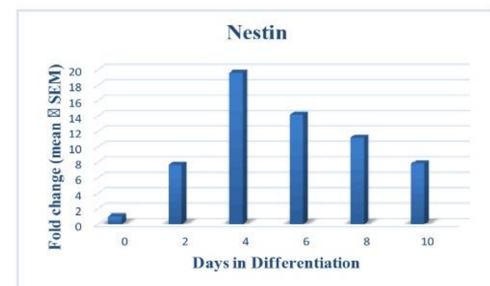
4.1.1 Characterization of mouse neural cells

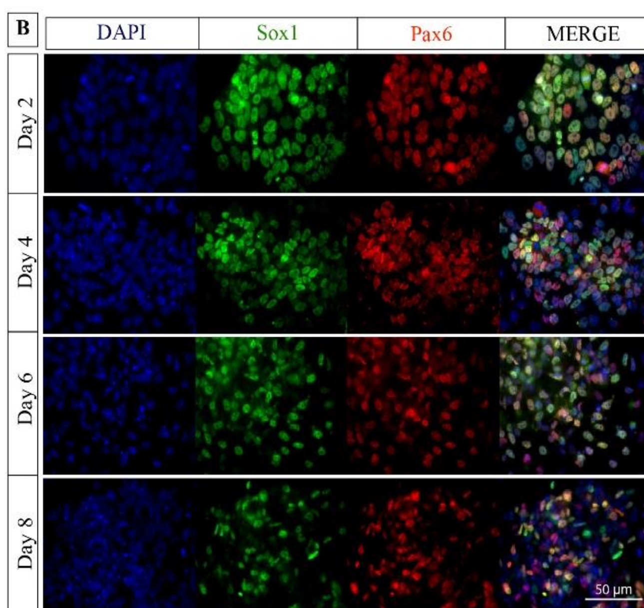
Here we demonstrate that neuroectodermal lineages can be derived from mouse ESCs and their efficiency are confirmed by the expression of molecular markers. The differentiation was monitored in 48 hours intervals, and gene expression (qRT-PCR) and protein expression (ICC) data were collected to understand the neuronal commitment during the differentiation of pluripotent mouse cells *in vitro* culture. The results of the immunostainings showed that undifferentiated mouse ESCs expressed the pluripotency markers OCT4, which dramatically decrease after the course of neural induction (Figure 8 A). However, within 2 Days of neuronal differentiation, they subsequently express neuronal progenitor markers: Nestin, Sox1 and Pax6 (Figure 8A and B). Then the neuronal population Tubb3 and Map2 was gradually increased from Day 6 onwards, thus leading to an efficient generation of NPCs Figure 8C.

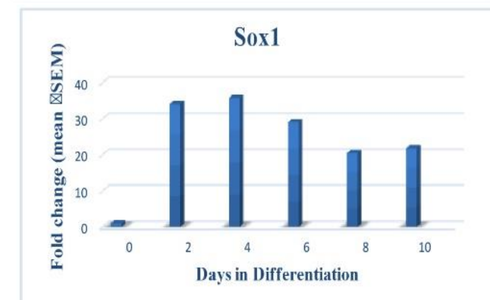
Next, we investigated the neuronal expression on mRNA level. Quantification of q-PCR revealed a subsequent increase in neuronal progenitor markers (*Nestin*=7.661 to 19.612(±0.78 to ±1.58 SEM), *Pax6*=21.958 to 72.086(±3.73 to ±11.0 SEM), *Sox1=20.45* to 35.83(±1.21 to ±4.28 SEM) from Day 2 to Day 10. In addition, we observed a steady increase in neuronal population after two weeks of differentiation (*Tubb3*= 43.483(±11.27 SD) and *Map2*=45.63(±13.64 SD). Taken together, these results suggest that (i) Sox1 overexpression maintains cells at the neuroepithelial stage and prevents their differentiation into radial glial cells, and (ii) Pax6 expression in neuroepithelial cells enhances their differentiation into radial glial cells and (iii) Nestin, Tubb3 and Map2 expression ultimately into neurons. To conclude our demonstrated that mouse ESCs have the ability to generate NPCs and differentiate further into neurons through monolayer culture system.

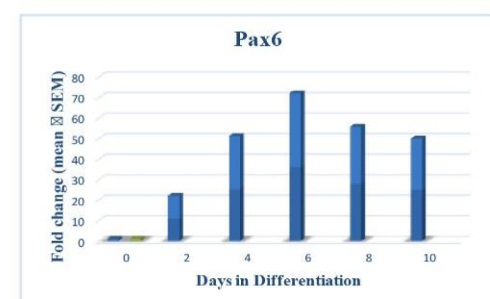












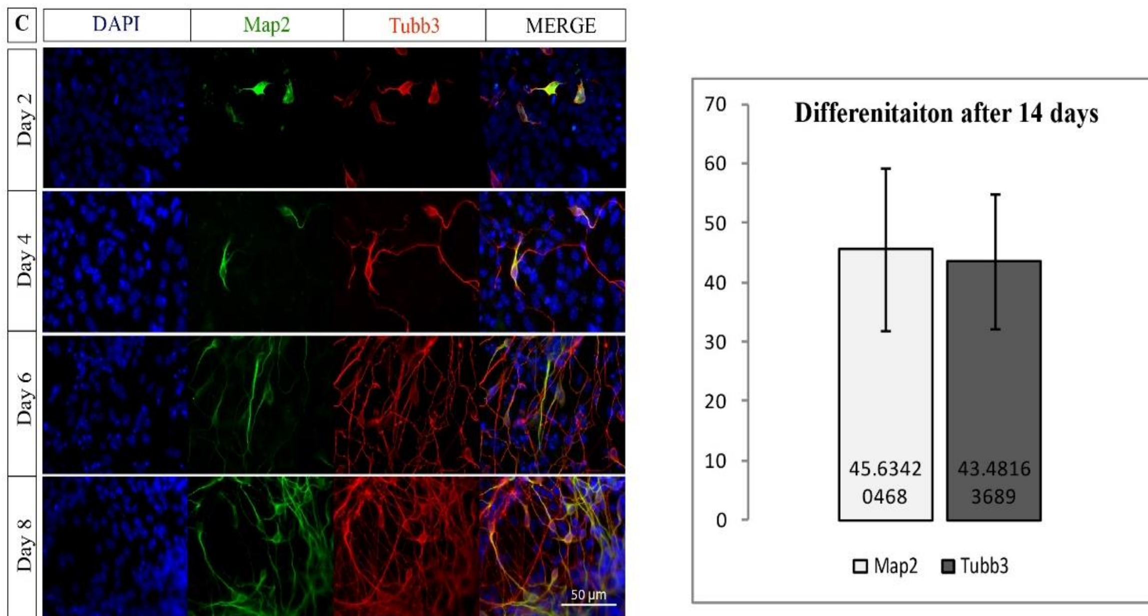


Figure 8. **Expression patterns of mouse embryonic stem cells both in an undifferentiated state and differentiated state (HM1 cell line) (A)** *Oct4* expression was observed in the initial phase of differentiation however, the expression level was down regulated over time. In contrast *Nestin* was up-regulated; **(B)** illustrates the expression pattern of Sox1, Pax6; **(C)** illustrates the expression pattern of differentiated neurons *Map2* and *Tubb3* Expression values were normalized to mouse *GAPDH* (reference gene).

4.2. Characterization of novel human iPSC lines

At the beginning of our experiments, in our laboratory, we derived novel human induced pluripotent stem cell (iPSCs) lines by somatic cell reprogramming, by the introduction of pluripotency-related transcriptional factors OCT4, KLF4, SOX2, and cMYC, as first reported by Yamanaka and his co-workers (Takahashi *et al.*, 2007). The schematic flow has been depicted in Figure 9A. The four "Yamanaka reprogramming factors" OCT3/4, SOX2, KLF4, and c-MYC were delivered into peripheral blood mononuclear cells (PBMCs) using the integration-free Sendai virus gene delivery method (Itskovitz-Eldor *et al.*, 2000; Yang *et al.*, 2008). In our work, the blood samples were collected from either healthy volunteer individuals or diseased patients, with full consent. 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). The generation procedure is published earlier by our team (Nemes *et al.*, 2016; Ochalek *et al.*, 2016; Táncos *et al.*, 2016) as detailed under Material and Methods section 3.3.

Novel iPSC lines were characterized first to prove their pluripotency and investigate their differentiation capacity. Without these characterizations, the cell lines could not be used in further studies. First, the phenotypic characteristics were studied. The expression of specific pluripotency markers was analyzed using conventional ICC staining. For phenotypic characterization OCT-4, NANOG, E-CADHERIN, and alkaline phosphatase were used; the DL-2 iPSC line was used for characterization and the results have been illustrated in Figure 9B.

Second, the Sendai virus free statuses of the cell lines were tested. Beginning with passage 5 of the iPSCs the absence/presence of Sendai virus vector was analyzed by RT-PCR using Sendai virus vector (SeV) - specific primers. After 7 passages, the elimination of the reprogramming vector was confirmed in BIOT- iPSC lines, which were selected for further analysis Figure 9B.

Third, the differentiation capacities of the cell lines were tested. The key to the scientific and therapeutic potentials of human stem cell lines is their capacity to generate cells representative of all three human germ layers (endoderm, ectoderm, and mesoderm), and thus, their potential to generate all the tissues of the human body – pluripotency. In this work, all our lines were checked for all three germ layers. The expression of germ layers was analyzed using the following germ layer markers (GATA4-Endoderm, Brachyury-Mesoderm, and TUBB3-Ectoderm) Figure 9B. Moreover, finally, another key requirement for hPSCs is a diploid karyotype (46: XY for male and 46: XX for female) was investigated by standard clinical karyotyping (karyotyping data not shown).

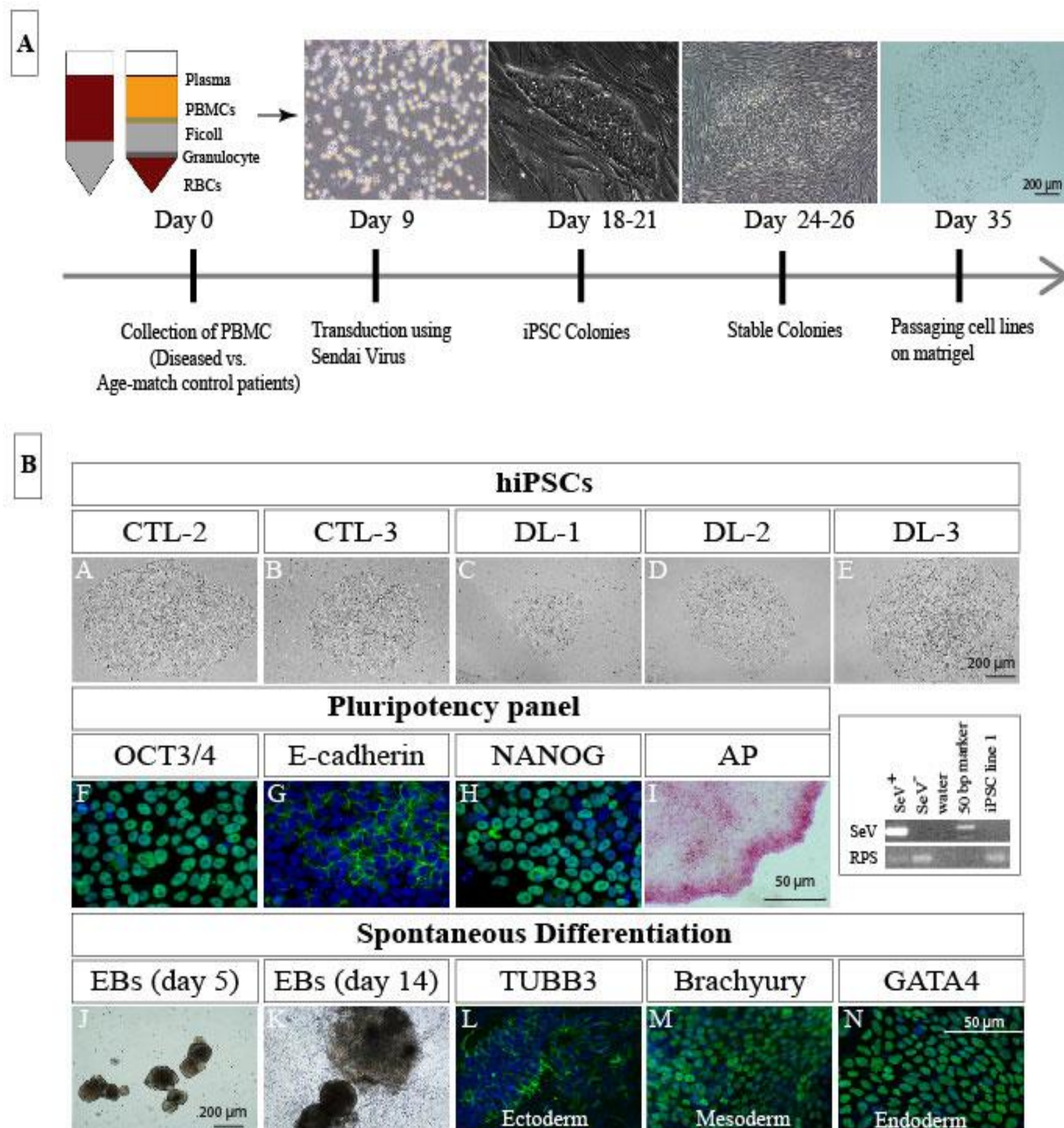


Figure 9. Derivation and characterization of pluripotent stem cells from human peripheral blood cells. **(A)** Overview of human pluripotent stem cells derivation **(B)** Characterization of human DL-2 pluripotent stem cells Chandrasekaran *et al*., 2016. First Row: Morphology of generated hiPSCs (5× lines), Second Row: Representative immunofluorescent micrographs of iPSCs positive for stem cells markers OCT3/4, NANOG and E-CADHERIN (in green). Alkaline phosphatase (AP) activity was also detected in iPSCs. RT-PCR was analyzed for the absence of Sendai virus vector (SeV) using SeV specific primer. Ribosomal protein subunit S-18 (RPS-18) expression served as normalization controls for the RT-PCR, Below Row: Spontaneously formed EBs in suspension culture (Day 5) and after attachment and further differentiation (Day 14). Immunostainings for endodermal (GATA4), mesodermal (BRACHYURY) and ectodermal (βIII-TUBULIN) germ layers, markers (in green). Nucleus is labeled with DAPI (in blue).

4.3 Differentiation of human iPSCs to neuronal precursor cells

The ability to differentiate, culture, and manipulate neurons is of tremendous interest to laboratories seeking to study human neurodevelopment and neurological diseases. Based on our results of mouse ESC differentiation, we went on to generate, compared different neuronal induction protocols and determined its effect on differentiation potential on human iPSCs. As 2D neuronal differentiation were effective in mouse studies, we set a direct comparison of 2D and 3D neural induction method in human iPSCs as well, as detailed in Figure 10A and B.

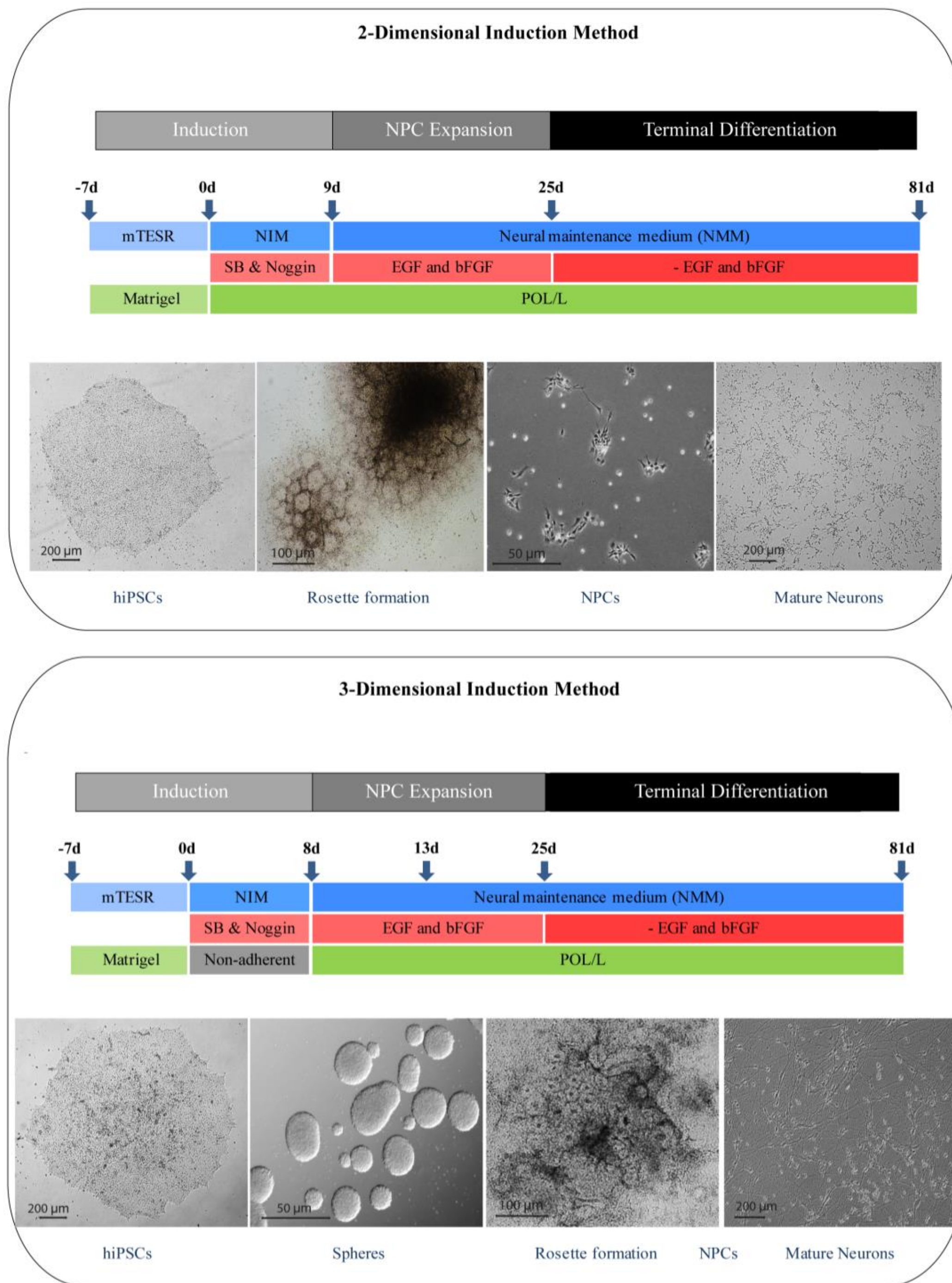


Figure 10. Comparison of the induction methods in hiPSCs. The figures show the experimental design of the neural induction methods of upper panel: 2D monolayer and lower panel: 3D aggregates. The protocol is described in detail under Material and Methods section.
SB, SB431542; POL/L, poly-L-ornithine/laminin; NPC, Neuronal Precursor Cells; EGF, Epidermal Growth Factor; bFGF, basic Fibroblast Growth Factor; d, Day; iPSC, induced pluripotent stem cells.

In the case of the sphere-based 3D neural induction, aggregates began to emerge around Day 4 following the neural induction. Following the sphere formation (between Day 4-8), the spherical aggregates were indistinguishable across the five different lines by gross morphological analysis Figure 11. Moreover, we did not observe significant differences in the efficiency of spheroid formation among the different hiPSC lines. As shown in Figure 12, the majority of the cells co-expressed NESTIN and PAX6.

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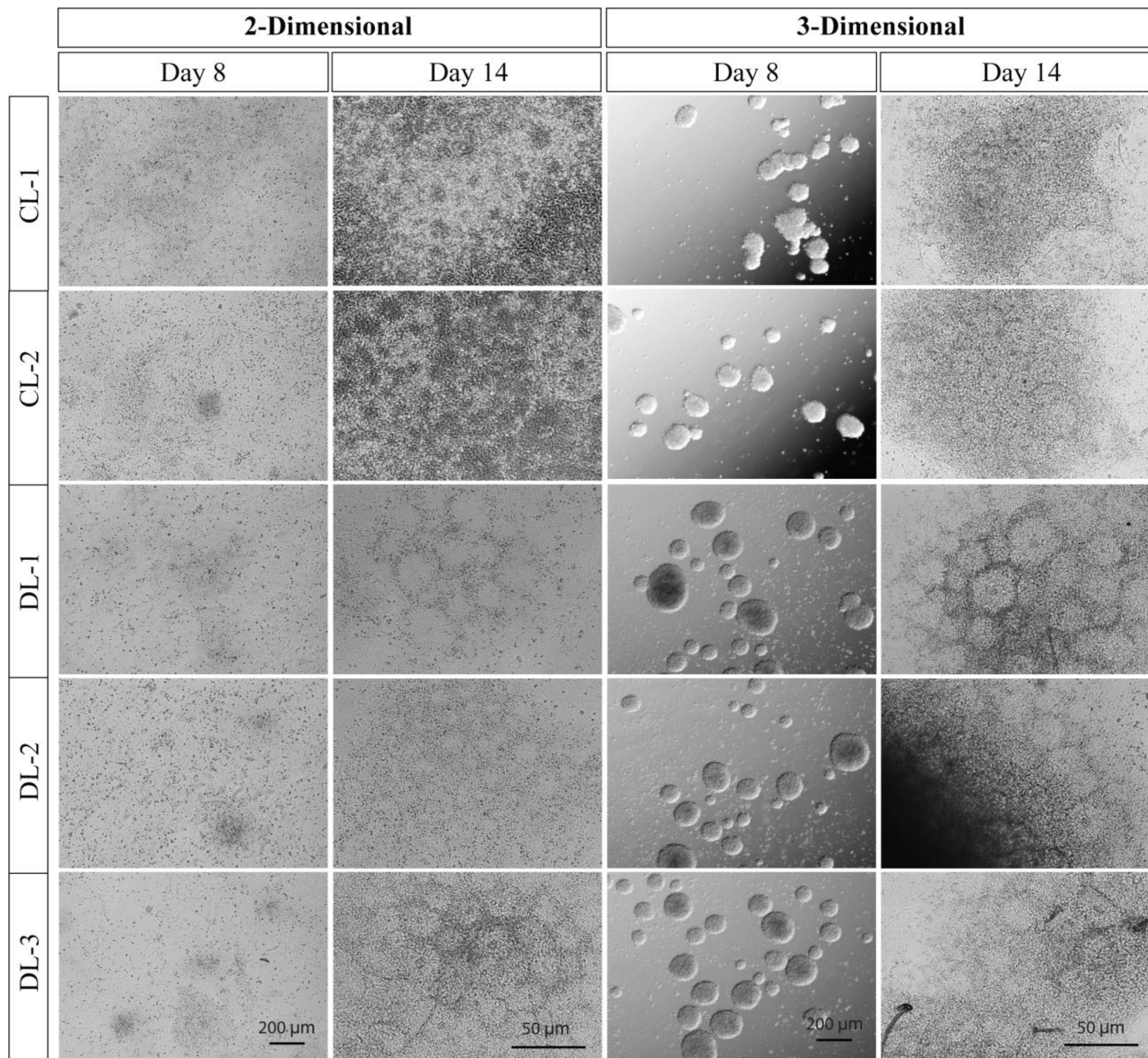


Figure 11. The progress of the two neural induction methods (2D vs. 3D) in hiPSCs. Phase contrast pictures of neural induction were recorded from live cells. The rosettes started to appear at Day 14 in both the induction methods. The desired neural rosettes containing NEP cells were manually picked under a binocular microscope under sterile conditions. They were plated en bloc in POL/L-treated culture dishes in Neuronal maintenance medium containing EGF (10 ng/ml), FGF-2 (10 ng/ml). Images recorded at Day 8 and Day 14.

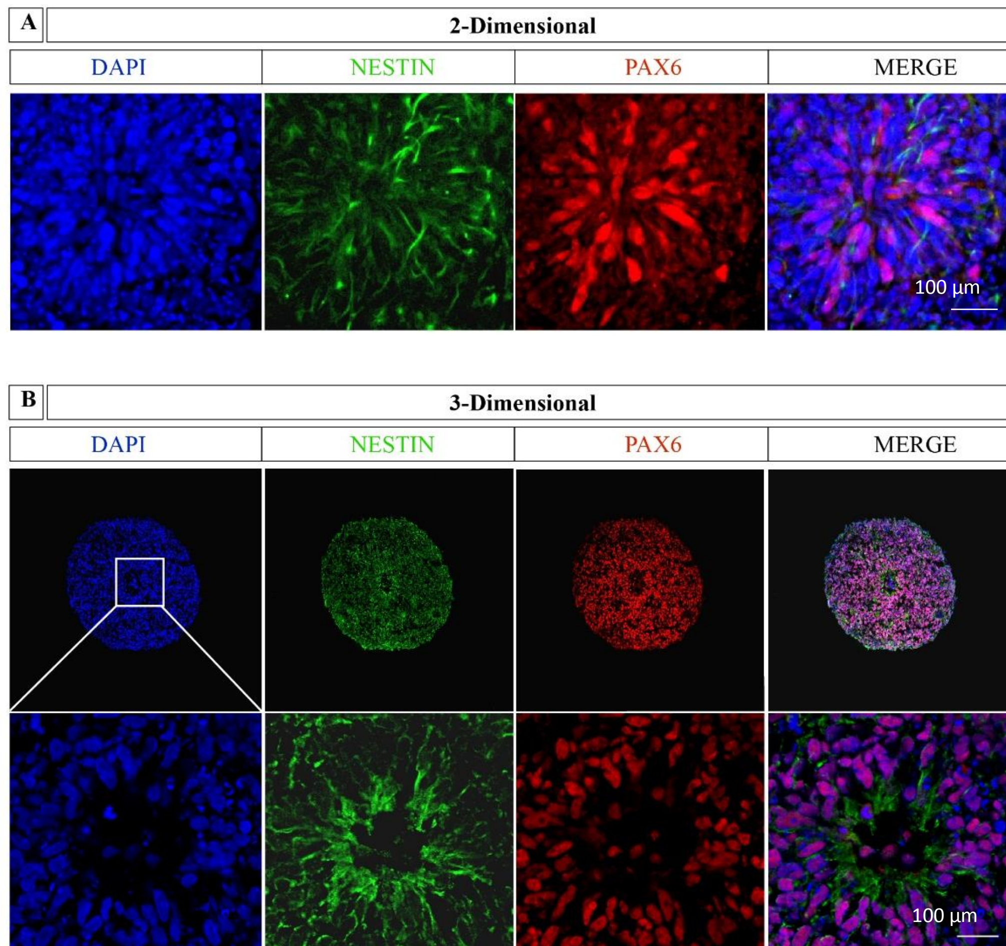


Figure 12. Immunocytochemical characterization of 2D and 3D neural induction derived neuronal rosettes. Representative confocal microscopic images of immunostained 2D neuroepithelial sheets grown on POL/L treated coverslips and 3D sphere cryosections. Neuronal progenitor cells forming neuronal rosette structures showed PAX6 (red) and NESTIN (green) positivity. ICC did not observe structural differences. Nuclei were counterstained with DAPI (blue).

Neural rosettes were observed in both induction methods independent from the investigated hiPSC line both with phase contrast (Figure 11) and confocal microscopy (Figure 12) or with electron microscopy (Figure 13). In semithin sections, we observed several neural rosettes in various sizes and shape (e.g. the number of cells forming the rosette), which we believe is connected to the age of the rosettes (dependent on which day during the induction they started to form). By their regular pattern, we called the area of the rosettes as zone I (Figure 13A and B). Central cavities contained cell debris or rarely intact cells (Figure 13C and D). Long, radially ordered cells surrounded the central cavity showed the ultrastructural features of radial glial cells, including cell coupling *zonula adherens* and tight junctions, deposits of glycogen storage granules and cilia (Kriegstein and Alvarez-Buylla, 2009) (Figure 13E and F). A network of smaller, multipolar cells among the rosettes formed a second zone (zone II) (Figure 13A and B). The cells in zone II contained smaller glycogen deposits and lipid droplets (Figure 13G). We observed mitotic cells both near the central cavities and further away from the center of the rosettes (Figure 13H).

In summary, early events such as neuroepithelial commitment and neural rosette formation of the cells were observed, structural changes were clearly distinguished and specific neuronal markers were already identifiable with ICC without detecting significant differences between the two induction methods or the different genetic background cell lines.

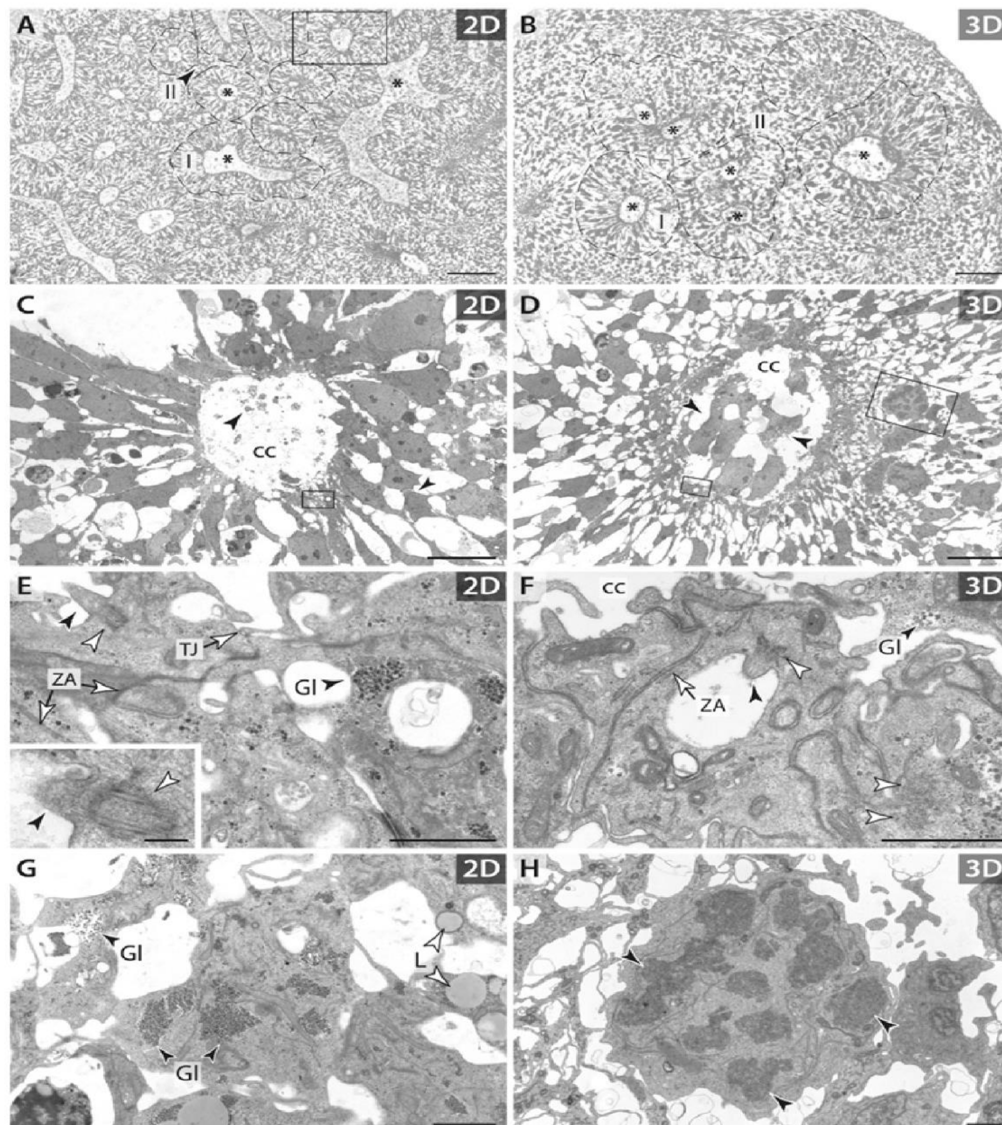


Figure 13: Ultrastructural characterization of CTL-1 culture derived with the 2D and 3D method. (**A-B**) Semithin sections represent neural rosettes with central cavities (*) surrounded by radially oriented cells (zone I). Cells among the rosettes form zone II. (**C-D**) Morphology of the rosettes on ultrathin sections at low magnification. The central cavity (cc) contains cell debris (panel C, black arrowheads) or intact cells (panel D, black arrowheads). Processes of narrow, radially oriented cells border this cavity. (Panel C is the magnified area of panel A.) (**E-F**). Enlarged areas of panel C and D show surface of the central cavities: cell coupling structures, i.e. tight junctions (TG) and *zonula adherens* (ZA), cilia (black arrowheads), basal bodies (white arrowheads) and glycogen deposits (Gl) can be seen in the cell processes limiting the central cavity (cc). Inset E: basal body (white arrowhead) and cilium (black arrowhead) in longitudinal section. (**G**) Cells further away the central cavity contain glycogen deposits (Gl) and lipid droplets (L). (**H**) A mitotic cell with condensed chromatin (black arrowheads) (scale bars: A-B: 50 mm, C-D: 10 mm, E-H: 1 mm, E inset 100 nm)

4.3.2 A higher proportion of PAX6^{+}/NESTIN^{+} neural progenitor cells are generated using 3D neural induction.

Following 4 passages of the rosettes, the morphology of NPCs was assessed by phase contrast imaging and for expression of varying neural lineage markers by ICC. Continuous passaging resulted in NPCs proliferating on a monolayer surface; however, cells from both methods continued to form rosette-like structures during the cell passaging (Figure 14).FACS analysis showed that the proportion of NESTIN and PAX6 double positive cells in 2D was over 85% (±0.822 SEM) and in 3D was over 93% (±0.796 SEM) ($p<0.05$) (Figure 15) (Supplementary Table 3). When tested for statistical differences between both methods, there was an average of 8% (±0.026 SEM) difference between the two induction methods with a significant increase ($p<0.05$; Supplementary Table 3) in 3D cultures. Thereby indicating 3D was superior to 2D. Quantitative RT-PCR showed that there was a clear difference in up-regulation of *NESTIN* expression in mRNA level in disease line vs. control lines, whereas up-regulation was inconsistent for *PAX6* in mRNA level (Figure 16). This result demonstrates that 3 out of 5 lines had only very modest increase in the 2D neural induction method, suggesting the 3D neural induction method is superior in mRNA level. Of particular interest was the presence of two PAX6 positive cell populations with both methods. One population was strongly positive for PAX6, while the other population expressed only weak PAX6 (Figure 14). This is in concordance with our previous study where we observed similar differences in NPCs derived from hiPSCs (Zhou *et al.*, 2016). Taken together, the overall results indicate that the proportion of PAX6^{+} and NESTIN^{+} NPCs was significantly higher on using the 3D neural induction method.

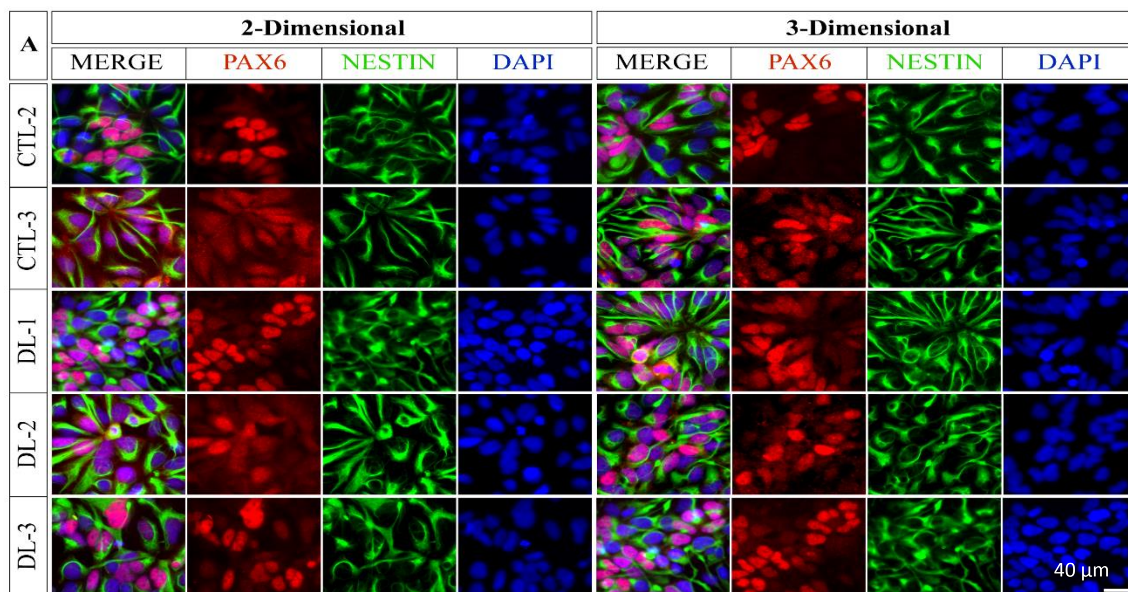
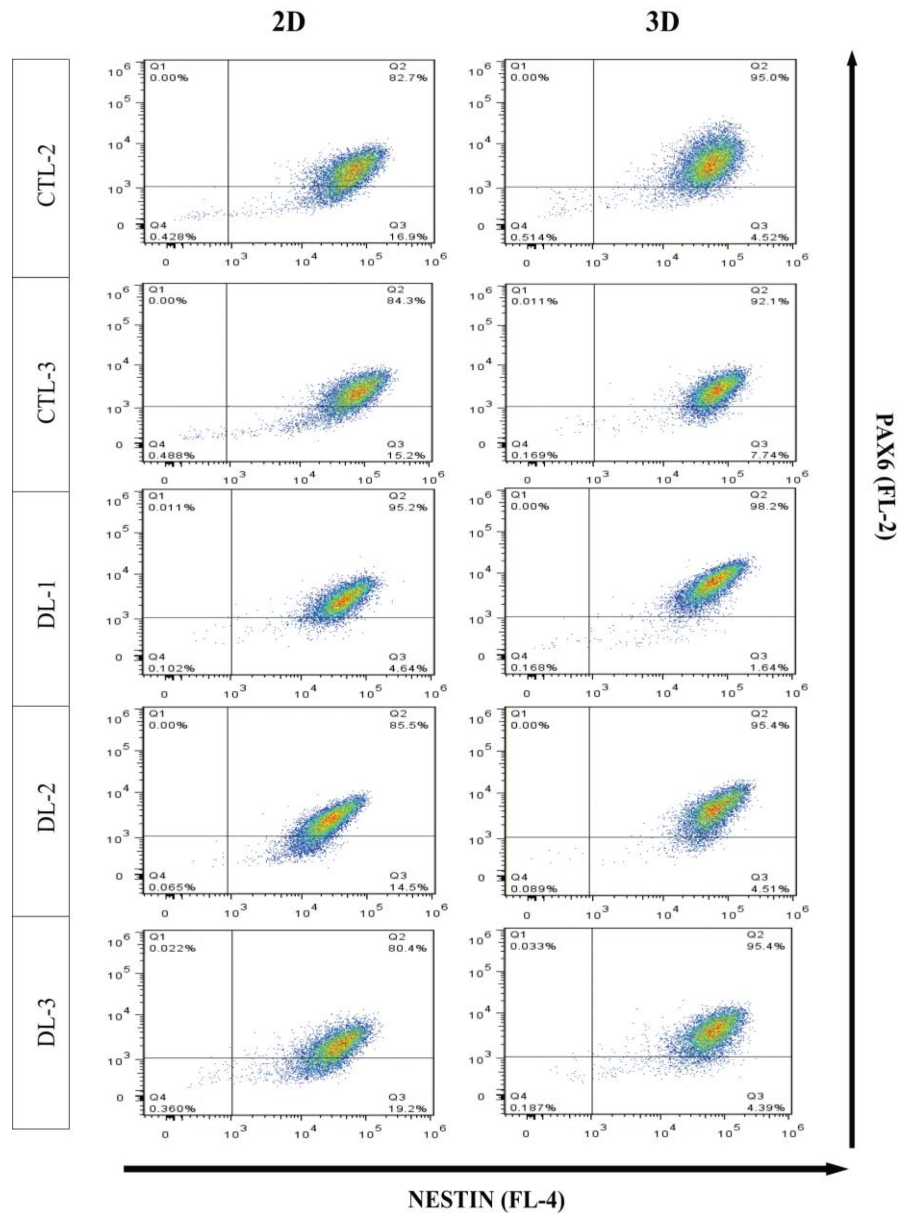


Figure 14: Comparison of hiPSC derived NPC populations generated with 2D or 3D neural induction methods. Immunocytochemical analysis of NPCs after induction. Neural progenitor markers, PAX6 (in red) and NESTIN (in green). Nuclei are counterstained with DAPI (in blue). Cells used for this experiment (ICC, FACS, and qRT-PCR) were analyzed in P4 and P5.



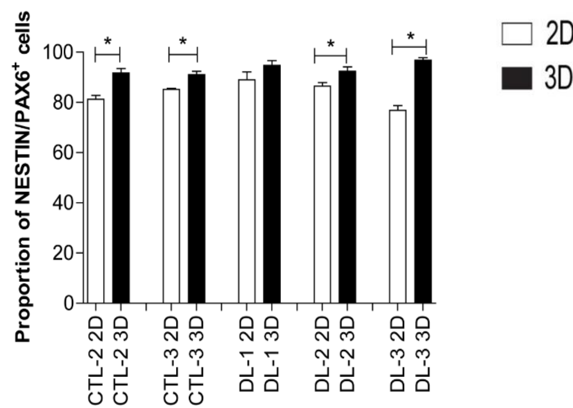


Figure15 Flow cytometer dot plots demonstrate PAX6 and NESTIN expression in a quantitative manner. The proportion of $PAX6^+$ and $NESTIN^+$ NPCs was significantly higher when the 3D neural induction method was applied, independent of the genetic background of the cell lines used (see details in Supplementary Table 3). From the dot plot Q1: $PAX6^+$ cells; Q2: $PAX6^+$ and $NESTIN^+$ cells; Q3: $NESTIN^+$ cells; Q4: $PAX6^-$ and $NESTIN^-$ cells.

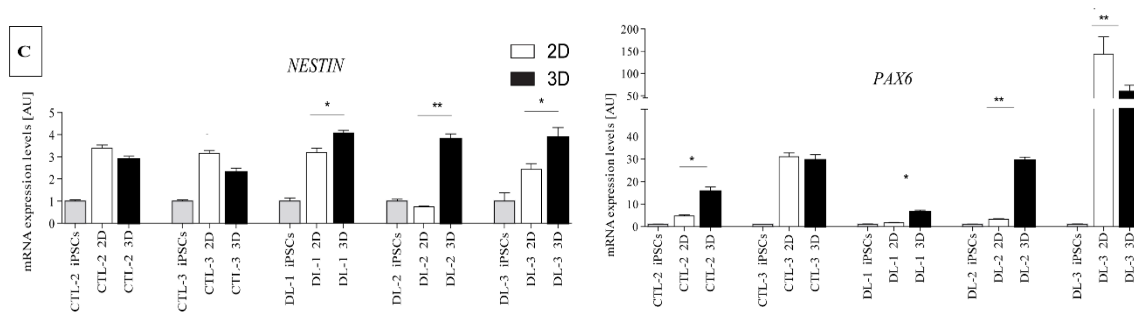


Figure 16 The qRT-PCR analysis presents *NESTIN* and *PAX6* expression of cultured 2D and 3D NPCs. Expression values were normalized to *GAPDH* (reference gene). Test for a significant difference (*$p < 0.05$) using Student's *t*-test; one-way ANOVA with a Tukey's post-test. The bars represent the Mean ± SEM of 3 independent cultures set. Cells used for this experiment (ICC, FACS, and qRT-PCR) were analyzed in P4 and P5.

4.3.3 The 2D neural induction increases the expression of SOX1 while SOX9 is unchanged

To investigate the neural fate of the NPCs, we investigated the expression of the NPC marker, SOX1 and the neural crest marker, SOX9. In both induction methods, the passage five (p5) NPCs maintained their expression of SOX1, but a subpopulation was positive for SOX9 (Figure 17). Our quantitative RT-PCR result demonstrates that there is a high population of cells that expressed *SOX1* expression while a low population of cells expressed *SOX9* expression (Figure 18). One cell line (DL-3) showed an inverse correlation between the mRNA level and protein level. The quantification revealed a strong cell line-related variation between the patients vs. control line in regards to the neural induction methods (Momcilovic *et al.*, 2016). Next, we quantified the SOX1 and SOX9 population using FACS (Figure 19). The analysis showed three divergent populations from both culture methods. The proportion of $SOX1^+$ cells ranged from 77.3% up to 83.0% in the 2D neural induction (SEM±1.27 and SEM±2.72, respectively), while in the 3D neural induction the range was from 71.2% up to 81.7% (SEM±1.19 and SEM±1.50, respectively). A similar distribution was observed in the proportion of $SOX9^+$ cells: ranging from 8.9-12.2% in 2D (SEM±0.896 and SEM±1.153) and 6.5-13.0% in 3D (SEM±0.793 and SEM±1.106) (see detailed data in Supplementary Table 3; $p<0.05$; Figure 18). There was a relatively sparse population of cells that were double positive for SOX1 and SOX9 (data not shown). Based on quantitative FACS data we can conclude that the 2D neural induction method generated more SOX1 positive neural progenitor cells in 4 out of the 5 examined cell lines.

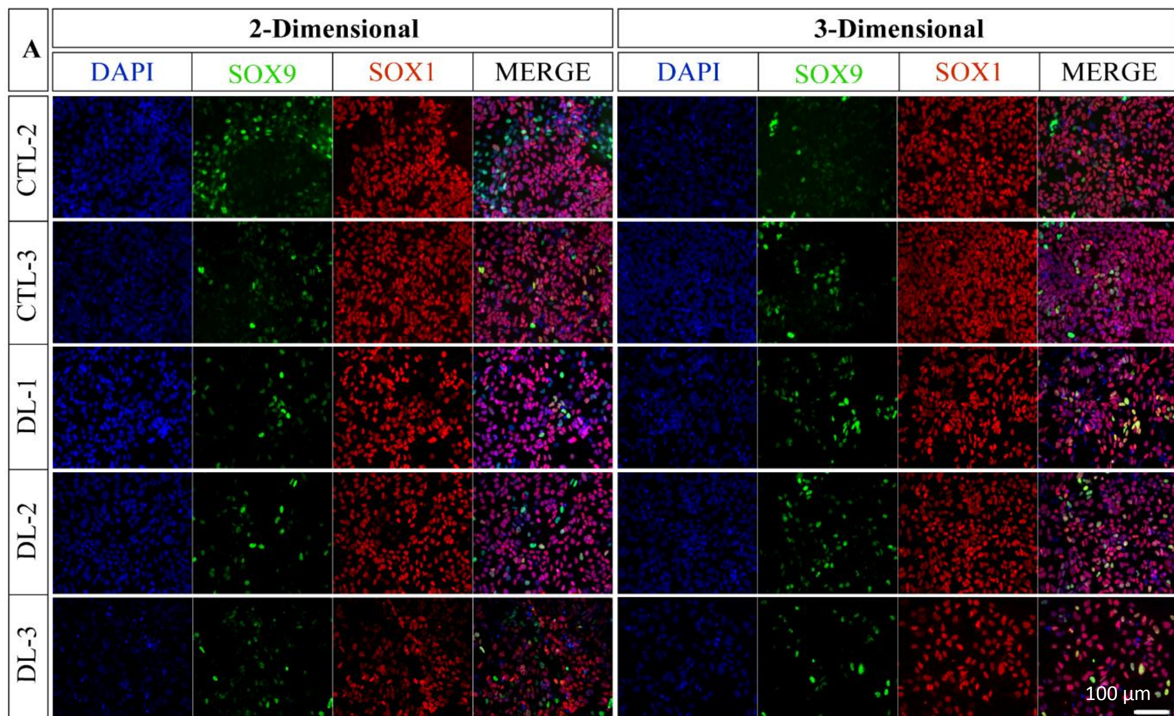


Figure 17. Characterization of neural progenitor's cells derived with 2D and 3D neural induction methods. Immunochemical analysis of SOX1 (in red) and SOX9 (green) populations in 2D and 3D neural progenitor's cells. Nuclei are counterstained with DAPI (in blue).

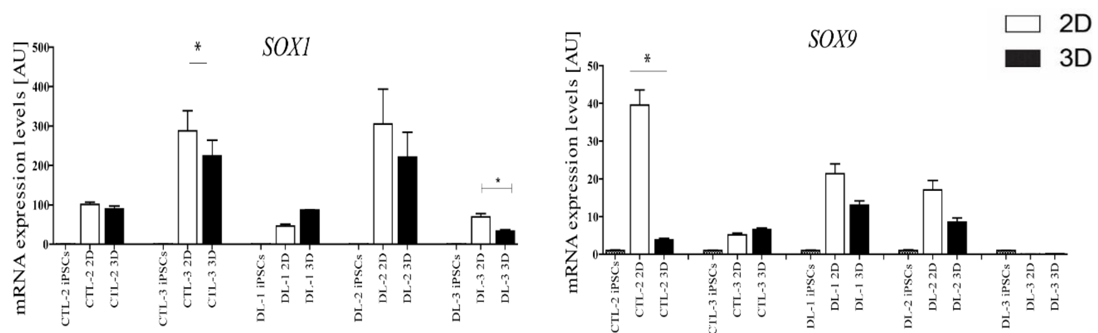


Figure18 qRT-PCR analysis presents the *SOX1* and *SOX9* expression of cultured 2D and 3D NPCs. Expression values were normalized to *GAPDH* (reference gene). Test for a significant difference (*$p < 0.05$) using Student's *t*-test; one-way ANOVA with a Tukey's post-test. The bars represent the Mean ± SEM of 3 independent cultures set. Cells used for this experiment (ICC, FACS, and qRT-PCR) were analyzed in P4 and P5.

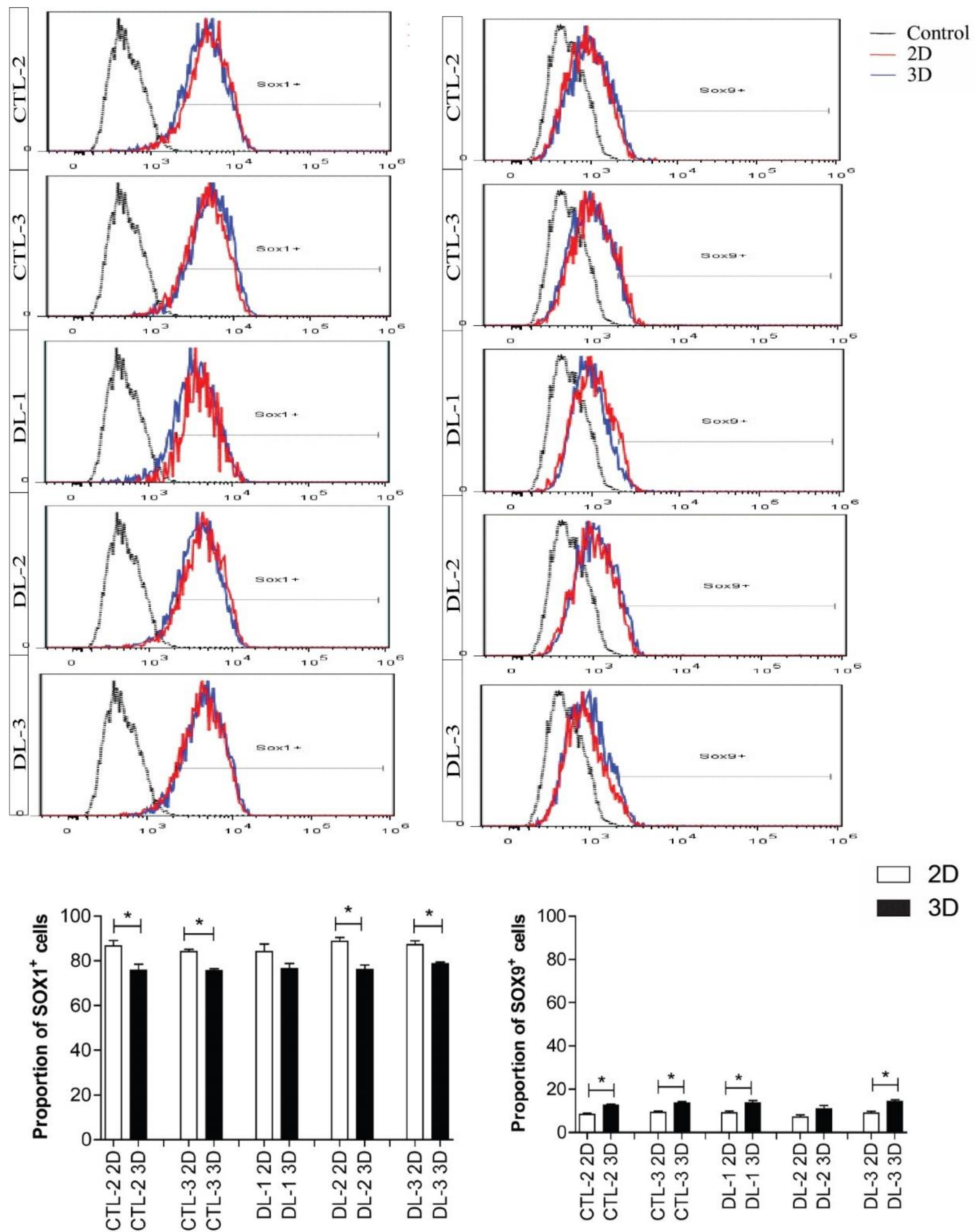
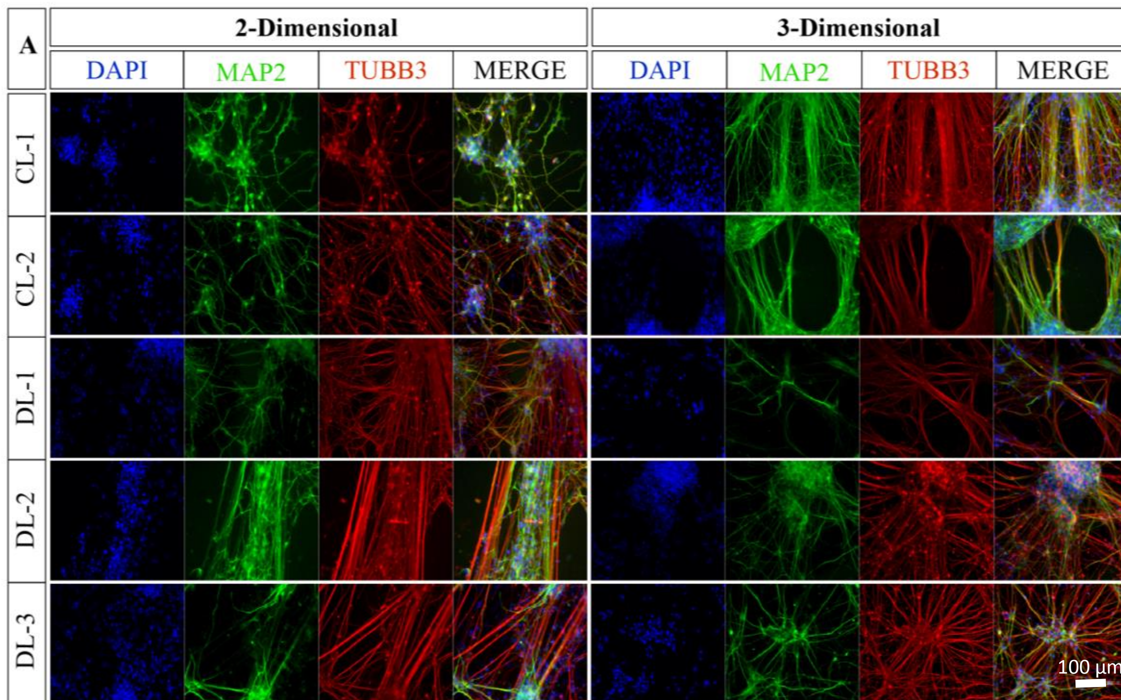


Figure 19 Quantification of SOX1 and SOX9 expression with flow cytometry. Specific gating was applied to quantify the SOX1 and SOX9 expression populations. The histogram demonstrated that SOX1 and SOX9 expression in 2D and 3D NPCs were more homogeneous.

4.4 Terminal differentiation of the NPCs revealed similar neuronal fate

We then evaluated neuronal fate by performing ICC and quantitative RT-PCR using varying neuronal markers. The ICC analysis showed that the majority of cells derived from the NPCs' from the two induction methods were positive for Tubulin Beta 3 class III (TUBB3), and microtubule-associated protein 2 (MAP2). These neurons also contained long neurites (Figure 20 A). As shown in Figure 20A, all the differentiated neurons exhibited fasciculation bundles. To

quantify and compare the expression level of several markers and their gene expression profile, qRT-PCR was performed on terminally differentiated neurons. As expected, a steady increase in the *TUBB3* (2D= up to 6-folds increase and 3D= up to 7-folds increase) and *MAP2* expression (2D= up to 11-folds increase and 3D= up to 12-folds increase) was observed (Figure 20B). Our qRT-PCR data showed that 4 out of 5 lines had a very modest increase in *TUBB3* and *MAP2* expression level in the 3D neural induction method at the mRNA level compared to 2D.



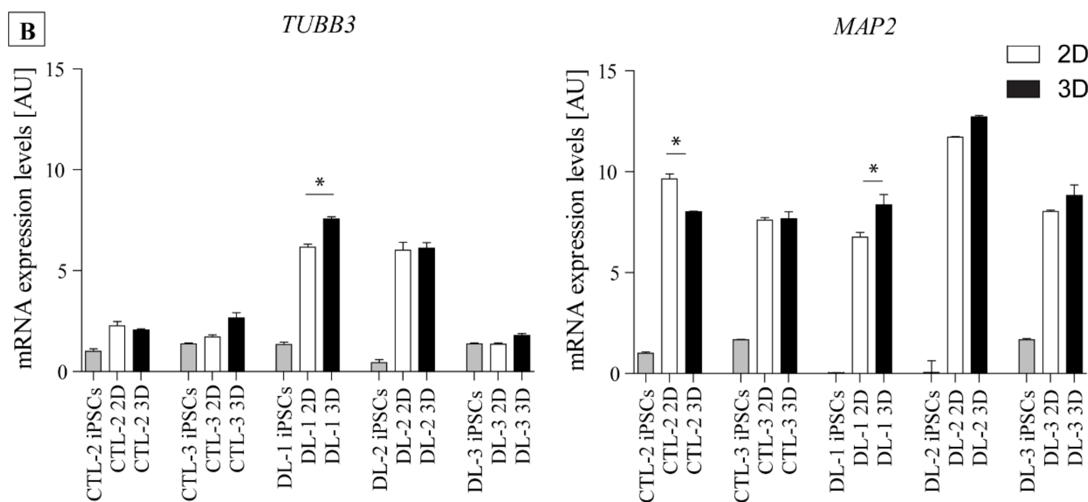


Figure 20: Terminal differentiation of NPCs after 35 Days revealed cortical neurons. (**A**) The representative immunocytochemical analysis shows MAP2 (in green) and TUBB3 (in red) in neurons differentiated from both induction methods. Nuclei are counterstained with DAPI (in blue). (**B**) qRT-PCR analysis plots reveal the expression profile of terminally differentiated neurons from 2D NPCs and 3D NPCs. The expression was normalized to *GAPDH* and *B2M*. Values were calculated as the relative amount of mRNA versus expression values of differentiated cells. Test for the significant difference was analyzed using Student t-test ($p<0.05$).

We then examined the expression of the cortical layer markers *CUX1* (c*ut-like homeobox 1;* expressed in IV-II layer of late-born/ upper layer cortical neuron) (Nieto et al., 2004) and *TBR1* (*T-box, brain, 1;* labels the cortical neurons and are widely expressed in layer IV) (Hadjivassiliou et al., 2010). As shown in Figure 21A, a slightly higher amount of TBR1 positive cells [ranging from 2D: 9.34% up to 22.39% (SEM±0.93 and SEM±2.20), while in 3D: 7.03% up to 22.2% (SEM±0.96 and SEM±4.95), respectively] were found in the NPCs derived from both neural inductions as compared to CUX1 [2D=10.56% up to 14.23% (SEM±1.69 and SEM±1.15), while in 3D=7.16% up to 18.98% (SEM±0.86and SEM±5.60), respectively] (Supplementary Table 3; $p<0.05$).

Subsequently, we also evaluated the expression of the glial subtype markers glial fibrillary acidic protein (GFAP) and aquaporin-4 (AQP4) (Roybon *et al.*, 2013) at a late stage in differentiation (week 11). Based on our result, a large variation in the proportion of GFAP positive cells could be determined across both induction methods [2D: 3.12% up to 45.29% (SEM±0.12 and SEM±1.69), 3D: 5.27% up to 48.11% (SEM±0.75 and SEM±11.41, respectively]. In the case of AQP4 a large variation was also detected, but was generally lower than GFAP [2D: 1% up to 36.43% (SEM±0.23 and SEM±3.0), 3D: 6.60% up to 32.20% (SEM±1.90 and SEM±4.74, respectively] (Supplementary Table 3; $p<0.05$; Figure 21B). This result indicates that both neural induction methods promote the differentiation of cortical neurons as well as glia, but also results in potentially remaining radial glia, which also expresses GFAP. To conclude, cell line difference were relatively high and modest difference could be determined in neuronal fate when comparing the 2D and 3D neural induction methods; however, this needs to be clarified in future studies.

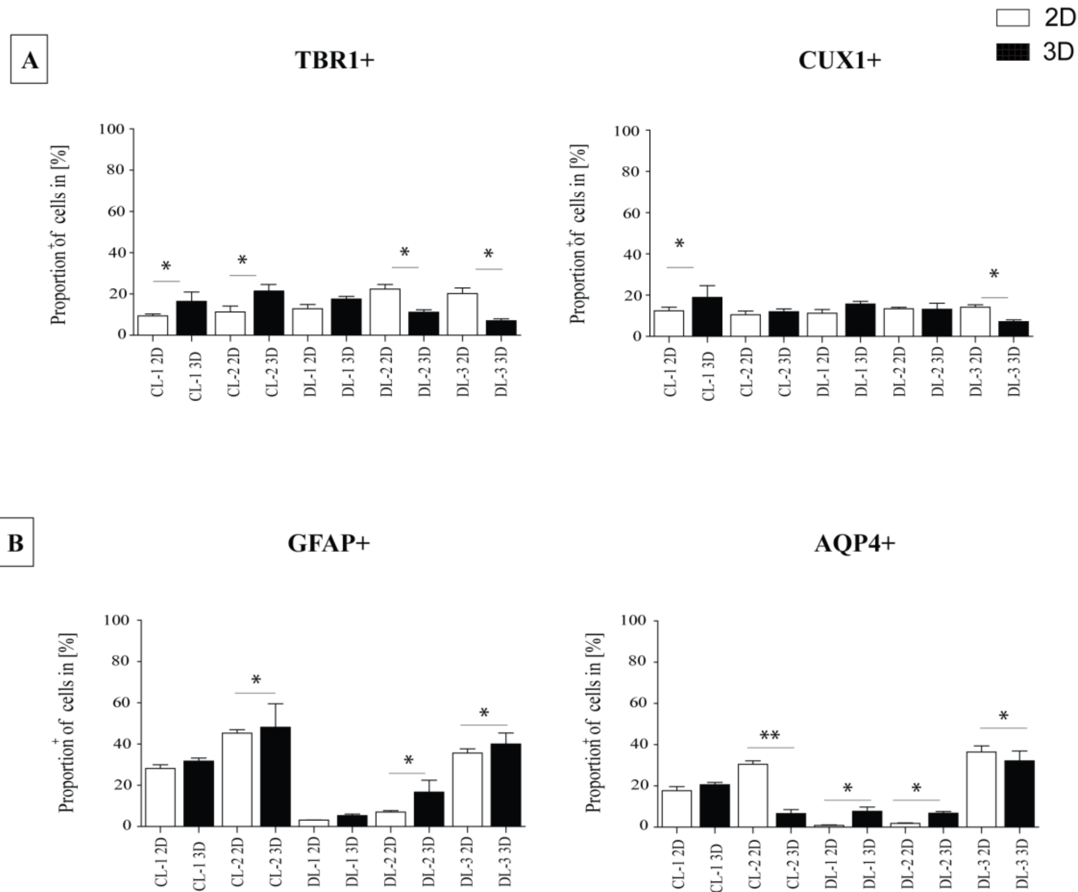


Figure 21: Characterization of neuronal cultures. (A) Quantification of TBR1/CUX/CULT1+ positive neurons derived from 2D and 3D neural induction. The proportion of cortical layers did not vary between induction methods. (**B**) Quantification of GFAP^{+}/AQP4^{+}astrocytes derived from 2D and 3D NPCs. Results were reported as Mean ± SEM of 15 fields from 3 independent cultures (*$p < 0.05$), using Student's *t*-test; by one-way ANOVA with a Tukey's post-test.

4.4.1 Neurite extension of neurons derived from 2D and 3D neural induction derived NPC cultures differs significantly

Axonal growth and branching are essential for the formation of the nervous system (Jiang and Rao, 2005). The neurite length of neurons derived by both induction methods was investigated by comparing the neurite outgrowth after five Days of terminal differentiation. At the time of plating, the pre-differentiated NPCs (Day 25) appeared spherical in shape with no apparent neurite outgrowth. About 24 hours post-plating, thin neurites began to emerge from the cell bodies of the cells. Five Days later, by the end of the differentiation period (at Day 30), many cells possessed between one and three neurites. Analysis of neurite length revealed the 3D neural induction derived neurons with longer neurites, ranging from 236.82 µm up to 461.16 µm (SEM±12.705 and SEM±25.21, respectively) while in the 2D induction method, neurites were from 213.35 µm up to 367.20 µm (SEM±13.36 and SEM±26.52, respectively). Cell line differences were also relatively high (Figure 22; Supplementary Table 3; $p<0.05$) and a remarkable and significant difference was observed within the differentiated neurites, suggesting the 3D method is more superior for neurite elongation.

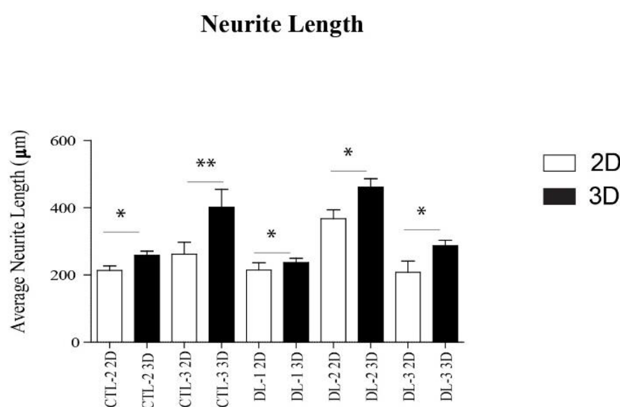


Figure 22: Characterization of neuronal cultures. (**C**) Bar graphs show neurite outgrowth measurements after 5 Days post plating. Neurite measurements show a significant enhancement in neurite length in 3D neural induction method that of 2D. Statistical significance was tested by an unpaired Student t-test (two-tailed). Results were reported as Mean ± SEM of 15 fields from 3 independent cultures.

4.4.2 Electrophysiology analysis

Functional analyses were deemed essential to verify the implications of the observation on longer outgrowth of neuronal cells derived by 3D neural induction compared to that of 2D neural induction-derived cells. Therefore, eight weeks old CL-2 neural cultures were analyzed with patch clamp to determine if spontaneous activation and electrophysiological properties were different among cell populations. Sodium and potassium channels were present in almost all cells analyzed (40 out of 42 studied cells). There was no significant difference in the sodium and potassium current amplitudes between the two induction methods. Peak amplitude of sodium currents was 1.97 ± 0.36 nA (range: 0.3 to 2.8 nA) for the 2D cultures (n = 7), and 2.26 ± 0.36 nA (range: 0.1 to 8.2 nA) for the 3D cultures (n = 35). Figure 23A and 23B shows examples for families of sodium currents evoked by the activation and the inactivation protocols, respectively. Examples of sodium currents from both induction methods are shown in Figure 23C. There was no significant difference between 2D and 3D neural induction-derived cultures in their spontaneous synaptic activity (observed in 4 out of 42 studied cells; an example is shown in Figure 23D), or in their LGIC-mediated currents. The presence of AMPA receptors was shown by currents evoked by kainate, which was observed in 13 out of 19 tested cells. Fourteen cells out of 21 responded to GABA (1333±392 pA), and 12 out of 17 responded to choline + PNU 120596 (749±219 pA). Examples of LGIC-mediated currents are shown in Figure 23E. In conclusion, spontaneous and evoked synaptic activity was detected in both types of cultures. There were no significant differences observed for the examined parameters, which would be in correlation with the induction method. Overall, both methods were suitable to generate mature, synaptically active neurons following terminal differentiation.

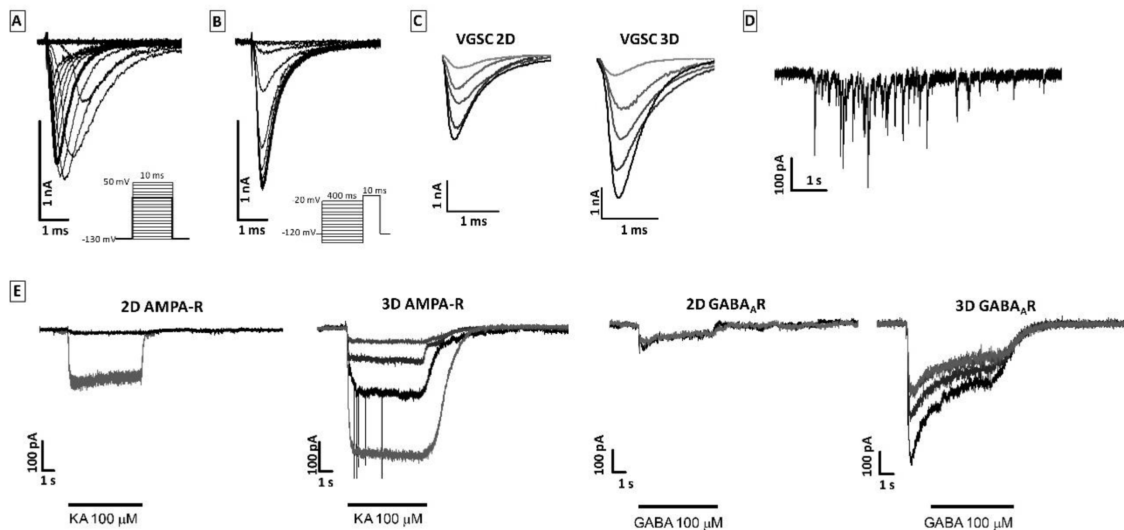


Figure 23: Ionic currents of neuronal cultures derived with 2D and 3D induction protocol from CL-2 iPSC line. (**A**) Example for currents evoked by the activation protocol. The inset shows the voltage pattern. The thick line indicates current evoked by a 10 ms step to 0 mV. (**B**) Example for currents evoked by the inactivation protocol. (**C**) Five representative examples for sodium currents from cultures differentiated by the 2D and 3D method. Sodium currents were evoked by depolarizations from -130 to 0 mV. (**D**) Example for the spontaneous synaptic activity observed in neuronal cultures. (**E**) Examples for LGIC-mediated currents from cells differentiated by the 2D and 3D methods.

4.5 Generation of Astrocytes from stem cells

The study of metabolic and functional features of astrocytes, in their resting state or different defined activation scenarios, faces a number of challenges: 1) measurements *in vivo* require a distinction of astrocytes from surrounding cells for the analytical endpoints chosen; 2) *ex vivo* studies, using e.g., FACS-purified adult astrocytes, suffer from a compromised viability of the obtained cells, and from indeterminate activation states, when put in culture; 3) *in vitro* studies mainly rely on studies of mixed populations, prepared from relatively immature cells. They may contain precursor cells, reactive astrocytes and other cell types, such as microglia. Additional approaches would thus be desirable to further explore astrocyte biology. In our study, we generate a population of mouse non-proliferative, non-activated astrocytes first and later on translate the same concept for human astrocyte generation from hiPSCs. The generation of mouse and human astrocytes has been illustrated in Figure 24A and B.

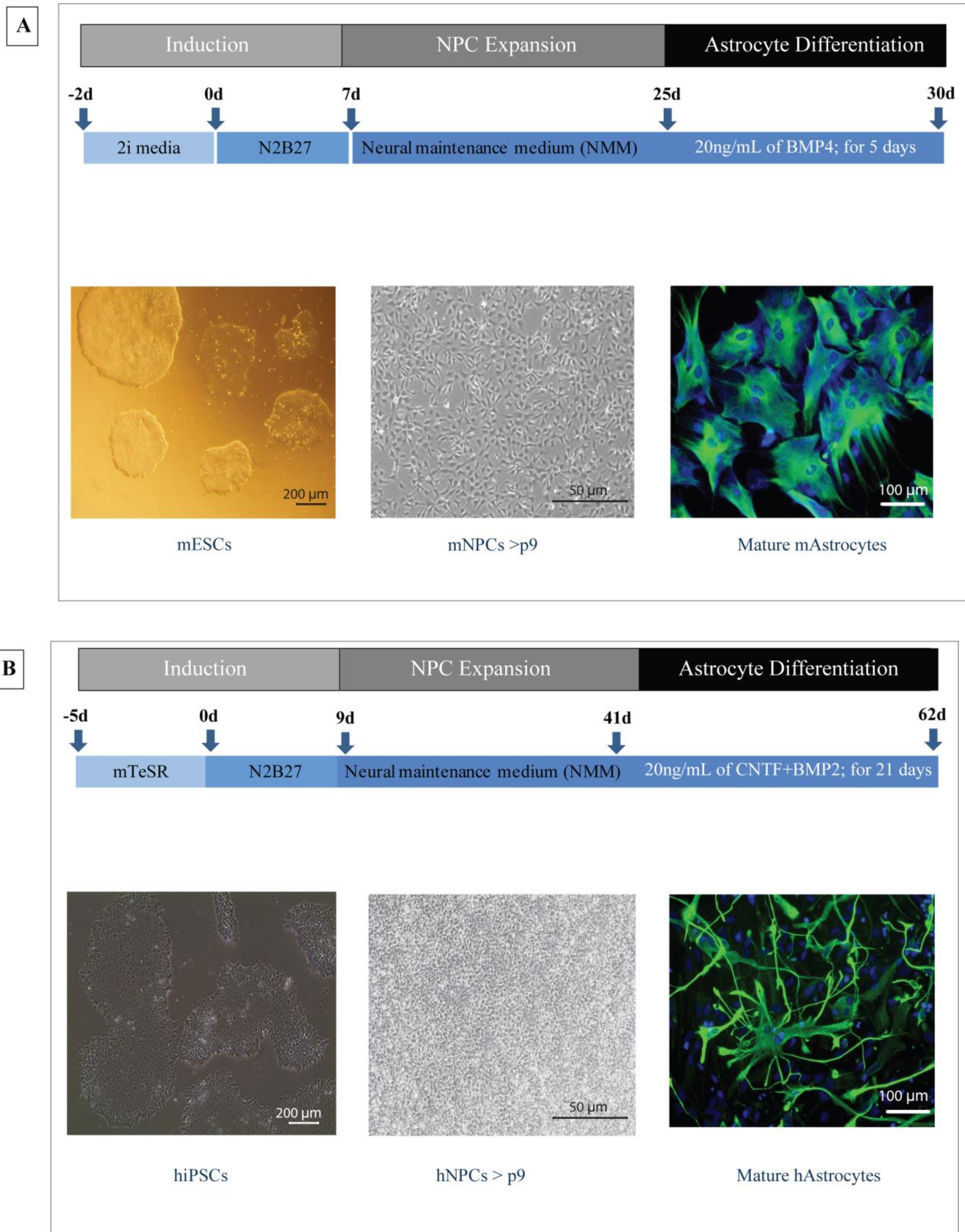


Figure 24. Astrocyte generation (A) The figures show the experimental design of the astrocyte production from mouse ESCs (B) human astrocytes from human iPSCs. *mESCs, mouse embryonic stem cells; iPSC, induced pluripotent stem cells. mNPC, Mouse Neuronal Precursor Cells; hNPC, Human Neuronal Precursor Cells; mAstrocytes, mouse astrocytes; hAstrocytes, human astrocytes; CNTF, Ciliary neurotrophic factor; Bmp4, bone morphogen protein 4; d, Day.*

4.5.1 Rapid generation of non-proliferating, mature astrocytes from mouse ESCs

Here in this study we use mouse embryonic stem cells to generate murine astrocytes; were these cells were differentiated in a three-step based procedure. Lesist's group (Kleiderman et al., 2016) implemented this method.

1) Embryonic stem cells were differentiated for 7 Days into neural stem cells of divergent populations when plated on gelatin-coated dishes in N2B27-medium without growth factors. The formed neural stem cell niche contained heterogeneous bipolar cells along with other cell types, in our experiments; we aimed to choose "bipolar like-cells" for NPC propagation. Bipolar cells are intermediate cells that are responsible for neuronal-glial formation, especially when treated with astrocyte cytokines like CNTF; they form mature astrocytes (Abbott, 2007)

2) The NPCs were maintained in EGF and FGF2-containing medium for several passages (until P8). To enrich the homogeneous population of bipolar cells, a selective trypsinization/mechanical cleaning was implemented (Kleiderman *et al.*, 2015).

3) The formed 'NSC' population from mouse cells was enriched into GFAP-positive astrocytes upon BMP-4 exposure.

From our mouse studies, we could show that >25% SEM±1.10 of the cells showed background staining just after 3 days of cytokines treatment (10 ng/ml of BMP-4) (Figure 25).

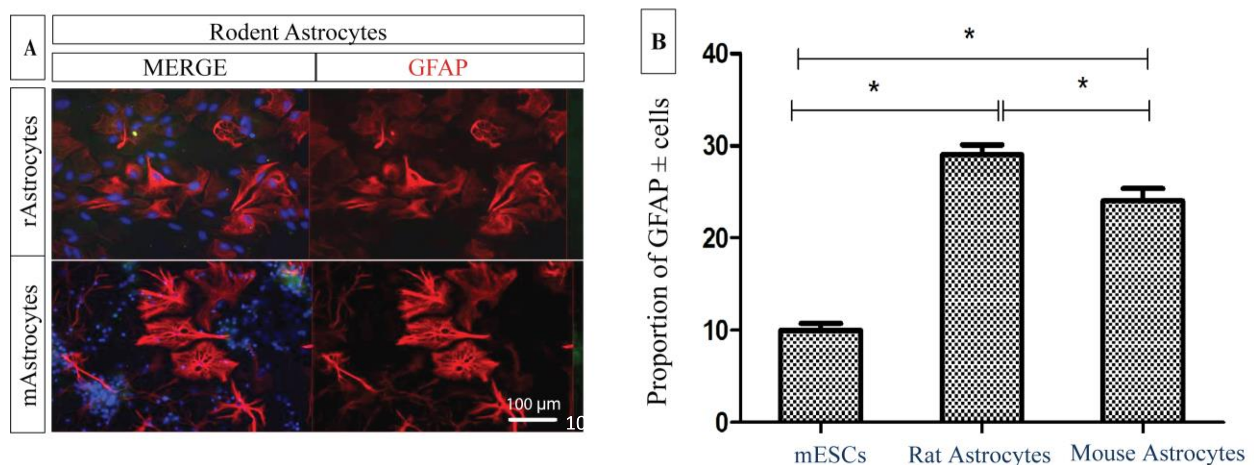


Figure 25. Mouse Astrocyte generation (A) ICC panel of GFAP$^+$ cells in rodents **(B)** Proportion of GFAP$^+$ cells in rodents. *GFAP, glial fibrous acid protein.*

4.5.2 Human astrocytes generation from hiPSCs

For human astrocyte generation from hiPSCs, we used the 'secondary NPCs from 2D neuronal induction' (high passage NPCs over p8/9). The secondary NPCs are the cells that accelerate the expansion of the neuronal population and switch to gliogenesis to produce astrocytes by known soluble factors: IL-6 and BMP proteins or cytokines (Miller and Gauthier, 2007). This concept was also published previously (Kleiderman *et al.*, 2015). Using the same principle, we generated about >85% SEM±16.12 of GFAP and AQP4 double positive cells within 5 weeks (Figure 26). Additionally, we observed a steady increase in terminally differentiated neurons after 3 weeks of culture (Data not shown).

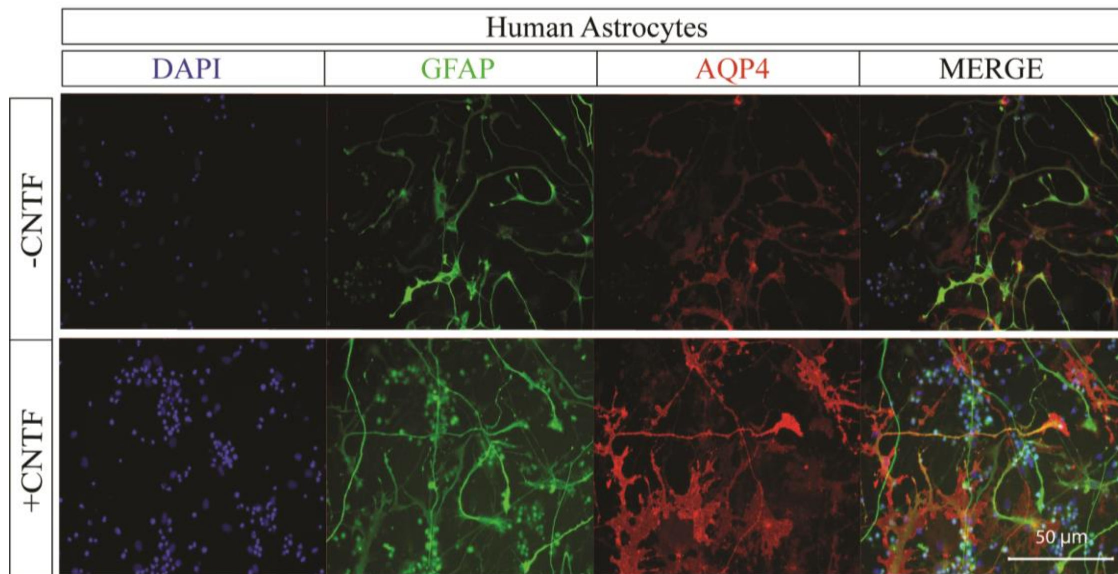


Figure 26. Human Astrocyte generation. Immunocytochemistry shows the AQP4^{+} and GFAP^{+} cells differentiated from hiPSCs, upper panel: -CNTF; lower panel: +CNTF. *CNTF; Ciliary neurotrophic factor.*

4.5.3 Comparison of mouse vs. human astrocyte protocol

The interspecies variation between human and rodent astrocytes underlines the need for authentic human astrocytes for disease modeling. Importantly, we observed a morphological difference between the rodent and human astrocytes as stated by (Oberheim *et al.*, 2009; Zhang *et al.*, 2016). To characterize the generated astrocytes, well-known markers such as GFAP and AQP4 were quantified on mRNA level by qRT-PCR (Figure 25B). Gene expression levels were normalized to GAPDH and B2M. The expression of mouse and human GFAP were compared with rat GFAP expression (which was determined as control; purchased from Thermo Fischer Scientific). Our results showed an up-regulation of both the astrocyte markers (AQP4 and GFAP) in all three species (rodents and humans). Additionally, the concept was proved by immunostainings as well (Figure 25A). The mouse protocol takes only 5 Days to obtain mature astrocytes, and the efficiency is high while the human astrocytes derivation required 5 weeks in the presence of CNTF + 2%FBS. Human cells differentiated in the presence of CNTF regardless of the presence of serum in culture media showed significantly higher levels of GFAP^{+} cells compared to the control NSC cells in NPC media Figure 26. The increase in GFAP positive cells did not coincide with the number of Tubb3 neurons. Astrocytes identify further validated with Aquaporin4^{+} cells. Due to time contrast further planned analyses were not performed. As a nutshell, we have evaluated the comparison of astroglial generation from both human and mouse depending on cellular morphology and gene expression profile. Thus, we conclude that generation of mouse and human astrocytes from pluripotent stem cells is possible with nearly similar efficiency.

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5. NEW SCIENTIFIC ACHIEVEMENTS

In this study, we applied *in vitro* cellular models in two mammalians, the mouse, and human to investigate the neural identity and neural fate of pluripotent stem cells by comparing 2D and 3D neural induction approaches. The following new scientific achievements were realized:

1. Both mouse ESCs and hiPSCs were efficiently differentiated towards neuronal lineage by dual SMAD inhibition method. In both species, the formation of neuronal rosettes in early NEP phase was detected and neuronal precursor cells (NPCs) were successfully generated and cultured further to generate post-mitotic neurons.
2. The 2D and 3D neuronal induction methods were compared, where the proportion of PAX6/NESTIN double positive NPCs was significantly higher when the 3D induction method was applied, independent of the genetic background of the cell lines.
3. The 2D and 3D neuronal induction methods were not significantly different in their main characteristics such as the differentiation rates of cortical neuronal subpopulations and the maturation and electrophysical activity of the generated neurons.
4. The newly generated human DL-2 iPSC line was characterized in detail, proving that the novel Alzheimer's disease patient-derived cell line is pluripotent and suitable for further studies. This is part of the first set of AD iPSC lines generated in Hungary.
5. A novel approach was used to differentiate mouse astrocytes from mouse embryonic stem cells for the first time in Hungary. The *in vitro* generated astrocytes were successfully characterized for their phenotypic features and at molecular levels for the GFAP and AQP4 mRNA and protein expression.

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6. CONCLUSIONS AND RECOMMENDATIONS

Small animal models, like mouse and rat, offer apparent advantages regarding high reproductive rates, low maintenance costs and the ability to perform specific experiments. However, the extrapolation of these results to human disease is often not straightforward, even with the usage of genetically modified animals. The demand for alternative methods for animal experiments has become increasingly strident in recent decades. As an alternative approach, "*in vitro* cell culture" can provide a reliable mechanistic insight of the disease without using a large number of animals. A recently developed technology by generating induced pluripotent stem cells provide a solid base for an outstanding technical and biological repertoire of approaches to effectively substitute animal models in several scenarios.
In our laboratory we had been investigating the methods of iPSC establishment, culture conditions and differentiation protocols with laboratory animal species such as rabbit (Táncos *et al.*, 2017) or mice (Nemes *et al.*, 2014). However, extrapolating these results to human disease was modest; therefore, we have now established this technology on human cells and investigate the translation of model system into human cellular models to gained insights. My current work specifies this translation in the field of neurobiology.

6.1 Neuroectodermal differentiation of mouse ESCs and translation to human iPSCs

The mESCs have been shown suitable to investigate differentiation and neurogenesis. I have shown that NPCs derived from mouse ES cells organize themselves into neuronal rosette-like structures, with an apico-basal distribution of polarity proteins similar to the *in vivo* neuroepithelial cells in the embryonic neural tube. Similar to the first neural precursors during development *in vivo*, ESC-derived neural stem cells exhibit an anterior identity (Dhara and Stice, 2008). Remarkably, one of the anterior markers – transcription factor PAX6 correspond to primitive neural epithelial cells that can differentiate into any region-specific neural progenitor (Zhang et al., 2010a). In summary, I have shown that mouse cells derived from such rosettes are capable of differentiation into mature neurons *in vitro.*

As described in the literature review section, mouse animal and cellular models can serve as a great *in vitro* model system for studying human disease, but for better translation into human medicine, an adaptation of methods to human cellular models is needed. As an emerging field of stem cell research, the use of iPSC-derived human neurons is off high demand especially for modeling neurological diseases such as Alzheimer's, Autism spectrum disorder and Parkinson's disease.

The 2D neural induction method was implemented to generate neural cells (Liu *et al.*, 2013). Although adherent neural induction had many advantages such as biocompatibility, controllability, and observability (Baharvand *et al.*, 2006), the cells lacked *in vivo* neural characteristics of the CNS. Due to variability in information between the induction methods, I, therefore, decided to compare both induction methods in detail to determine a versatile methodology for the generation of authentic, functional neurons from PSCs. Previously, other groups have described the generation of brain-derived NPCs from 3D-spheres and monolayer (Muratore, Srikanth *et al.*, 2014) where they presented their data on the maturation of neural progenitors and generation of functional mature neurons. However, none of the group went on to

compare two induction methods in parallel. My study describes the early stage of neural induction *in vitro* and cellular properties of human NPCs derived from human iPSC.

6.2 Efficient generation of neural cells from induced pluripotent stem cells

Our previous work with Zhou et al. demonstrated the NPC culture system by inhibition of BMP and TGF-ß signaling by Noggin and SB431542 (Zhou *et al.*, 2016). Also, Zhou et al. compared the regional identity of the neural rosette and specific neural subtypes of their progeny with a low and high concentration of bFGF and EGF in the culturing system. In her studies, results indicated that, in the propagation of low concentration of mitogen condition can promote forebrain cortical neurons while in a high concentration of mitogen condition can generate mid-hind brain neurons resulting in cholinergic neurons (Zhou *et al.*, 2016).

In my study, I have performed a direct comparison of 2D neural induction and 3D neural induction methods to assess which might be more efficient in the production of cortical neurons. The first successful induction of neurons from human PSCs was published by Zhang et al. in 2001 (Zhang *et al.*, 2001). Using Zhang's method of induction, important comparisons have been drawn between the rosette stage and human embryo development stage (indicating the neural tube formation vs. the third week of gestation) (Pankratz *et al.*, 2007). During neural induction, human PSC undergoes morphogenetic events to form columnar epithelial cells termed "neural rosettes" (Perrier *et al.*, 2004). These structures are capable of differentiating into various region-specific cells (neuronal and glial). My findings clearly demonstrated that the neural rosettes produced by the synergistic inhibition of dual SMAD induction (BMP and TGF-β) exhibited similar neural rosette formation when both methods were used. Thus, the formation of neural rosette *in vitro* recapitulates the neural tube formation *in vivo* (Muguruma and Sasai, 2012). Following ten days of neural induction, no morphological or qualitative differences in the ICC could be observed in the formed rosettes, suggesting a uniform expression of NPC markers in the NEP phase (such as SOX1, PAX6, and NESTIN) by both induction methods (2D and 3D).

Strikingly, my FACS analysis data showed a significant increase of PAX6/NESTIN positive cells in the 3D neural induction method, when NPCs were isolated from the rosettes. This indicates that the 3D neural induction method results in more radial glial and may potentially produce more forebrain neurons (Molyneaux *et al.*, 2007; Mariani *et al.*, 2012). PAX6 is a neuroectodermal marker (Zhang *et al.*, 2010; Onorati *et al.*, 2014) in the differentiating human CNS and is expressed in the dorsal forebrain, including a region that gives rise to the cortex and functions in patterning the brain (Osumi, 2001). A study by Suter et al. suggested that overexpression of PAX6 in noncommitted cells favor neural lineage commitment by differentiation into radial glial and subsequently into neurons (Suter *et al.*, 2009). Based on my results, I find 2D neural induction is easy to setup and faster to perform but generates less PAX6 positive cells, while 3D neural induction method is longer and more labor intensive, but generates more PAX6 positive cells.

To analyze differences in neural fate, I studied SOX1 vs. SOX9 positive cell populations. SOX1 is one of the earliest expressed pan-neuroectodermal transcription factor that appears before PAX6 (Pankratz *et al.*, 2007) and increases when NEPs begin to differentiate towards NPCs, within the embryonic neural tube (Ng *et al.*, 1997; Pevny *et al.*, 1998; Stolt *et al.*, 2003; Alcock *et al.*, 2009). It is also a marker for proliferating NPCs (Wegner, 2011). While SOX9 is also

expressed in NPCs and is important for its maintenance (Scott *et al.*, 2010), it is also expressed during neural crest stem cell differentiation (Stolt *et al.*, 2003; Wegner, 2011; Bergsland *et al.*, 2011), and thus may lead to slightly different progenitor subtypes like glial cells. Suter et al. suggested that overexpression of SOX1 in embryoid bodies leads to a large increase in NEP, RG cells and mature neurons (Suter *et al.*, 2009). Based on my quantitative FACS results, I determined the 2D neural induction method generate more SOX1 positive NPCs in the majority of the lines, indicating an early NEP formation compared to the 3D neural induction method, which does suggest a divergence in neural cell fate, however, this could not be verified following neuron differentiation. Furthermore, it has been shown in various publications that the upregulation of SOX1 expression results in the promotion of motor neurons (Shimojo *et al.*, 2015). Hence, I believe that the 2D neural induction method could be useful for the future generation of motor neurons (Du *et al.*, 2015) however, this needs to be clarified in future studies.

It has been shown that 3D culture is critical for sustaining the *in vivo* ontogeny of neurons (Blackshaw *et al.*, 1997; Ribeiro *et al.*, 2012). Previous studies have shown that 3D differentiation enhances the generation of neurons and neurite outgrowth (Frimat *et al.*, 2015; Sun *et al.*, 2016). Similar to these studies, our neuritic protrusion analysis demonstrated that neurons cultured with 3D neural induction generated significantly longer neurites, compared to the 2D neural induction. This observation is also in agreement with previously described findings (Koh *et al.*, 2008; Kraus *et al.*, 2015; Liu *et al.,* 2016). In this study, I have analyzed relatively young *in vitro* cultured neurons, due to technical limitations, which relate to the difficulty in analyzing longer projections in older and subsequently denser cultures. It was therefore not possible to measure the neurite length of terminally differentiated mature, electrophysiologically active neurons. Sparsely seeding neurons early in differentiation could be a way to overcome this issue in future studies, since it would be interesting to determine whether neurite outgrowth differences could also be observed at later stages in culture. Also, I have examined the effects of neural differentiation from hiPSC-derived 2D vs. 3D NPCs towards neurons. Based on the mRNA profiling and ICC, my results also indicated that 3D neural induction showed a modest increase in both MAP2 and TUBB3 in both groups, which is by previous findings (Hosseinkhani *et al.*, 2013).

I next sought to determine the proportion of cortical layer identity (TBR1 and CUX1) from these iPSC-derived neurons. My results showed that both conditions resulted in slightly more TBR1+ than CUX1+ cells, which indicates a preference in the generation of the superficial layer IV type cells over the other superficial layers. Although, I did not evaluate for the presence of the deeper layer neurons, the Shi protocol (that we based our differentiation protocol on), results in the production of both deep and superficial cortical layer neurons, with the deep layer neurons being born first (Shi *et al.*, 2012). It is also essential to note that our cultures of iPSC-derived neurons also contain RG, reflecting a heterogeneous environment that is more physiologically relevant to the development of the cells *in vivo* (Rooney *et al.*, 2016). This has been documented by several other groups regardless of protocol differences (Johnson *et al.*, 2007; Muratore *et al.*, 2014; Paşca *et al.*, 2015). My result also showed a line-to-line variability which might be related to the genetic background differences. Taken together, my results demonstrate that there were no intrinsic obstacles for generating different classes of projection neurons from hiPSCs using both

neural induction methods; however, the 3D method resulted in a higher proportion of NPCs with early born neurons that had longer neurites, indicating a slight preference for the 3D method.

Finally, I evaluated the functional characteristics of iPSC-derived neurons. I observed no significant differences between 2D and 3D neural induction methods in their spontaneous activity, or in their LGIC-mediated currents. Thus suggests that human neurons require longer periods to reach synaptic maturity *in vitro* culture conditions, consistent with other observations (Niu *et al.*, 2015). Our study also showed no significant difference between the DL lines vs. the CL lines that were similar to Momcilovic et al., where they showed no significant difference in the timeline of neural induction between the patient versus the control lines (Momcilovic *et al.*, 2016). Similarly, Schwanhausser et al. noted that not all mRNA equate with protein expression (Schwanhäusser *et al.*, 2011) that was observed in some of my cell lines.

6.3 Generation of astrocytes to promote cellular complexity and homeostasis by improving culture conditions

CNS neurons are never alone; they are often connected with astrocytes along with other cell types to form structural and functional networks. Astrocytes are the most abundant cell types in the central nervous system (Azevedo *et al.*, 2009) with a remarkable heterogeneity both in morphology and function. In the past, astrocytes were believed to act as “passive support cells” for electrically active neurons and to be primarily responsible for cellular homeostasis of the CNS, but current research shows their active participation in many other processes such as the formation of neural networks, recycling of neurotransmitters, and detoxification (Nedergaard *et al.*, 2003; Krencik and Ullian, 2013). Impairments in these functions, as well as physiological fluctuation in glutamate/K^+ levels, can trigger or exacerbate neuronal dysfunction (Zhang *et al.*, 2016).

Studies on the de-differentiation and neurogenic potential of astrocytes are scarce. This is mainly due to a lack of appropriate cellular systems. Here, in this study, I generated astrocytes from both mouse and human stem cells to investigate the pathomechanism of certain diseases in which they are involved. Using mouse stem cells, I was able to produce mouse astrocytes within a week, while with humans astrocyte was generated within 5 weeks. Though I managed to generate astrocytes from both cell types (mouse and human) allowing a direct comparison of morphology. A detailed comparative study is still required. Perhaps this needs to be clarified with secondary NPCs from 3D neural induction to determine neuronal fate commitment. Due to time constraints, co-culture experiments were not performed.

6.4 Keynote of this study

In this study, I applied an *in vitro* cellular model of two mammals, the mouse and human to study the neural identity and neural fate of pluripotent stem cells by comparing 2D and 3D neural induction approaches. Based on my results, it can be concluded that both induction methods are efficient to induce and produce NPCs, which can be differentiated into neurons and glial subtypes efficiently. This was true for both species, the mouse ESCs and different genetic background human iPSCs (healthy individuals and neurodegenerative disease patient’s) as well. However, 3D Neural induction promotes the efficient generation for more PAX6 positive cells and neurons derived from this method have increased neurite length, which is an essential feature for cell migration and RG-derived early neurons.

As a first part of the work, I focused on the production of pluripotent stem cells from human cells, using OSKM factors. The generated PSCs were characterized for pluripotency markers and differentiated towards the three germ layers. Here, I proved that the generated novel Alzheimer's disease patient-derived cell line is pluripotent and suitable for further studies (Chandrasekaran et al.: Stem Cell Research, 17, 78–80; 2016).

Next, I questioned how I could generate neuronal progenitor cells from stem cells; efficiently which method is suitable for disease modeling *in vitro* (Chandrasekaran et al. Stem Cell Research, 2017. Submitted).

The major findings of this work are the followings:

- Both mouse ESCs and hiPSCs were efficiently differentiated toward neuronal lineage by dual SMAD inhibition method.
- In both species, the formation of neuronal rosettes in early NEP phase was detected, and the relevant difference was observed between the species (mouse or human) or induction methods (2D or 3D neuronal induction. The possibilities and challenges of 2D vs. 3D models have been submitted for publication as a full paper (Poon et al. New Biotechnology, S1871-6784(16)32499-2; 2017).
- Neuronal precursor cells (NPCs) were successfully generated with both induction methods and cultured further to generate post-mitotic neurons.
- The proportion of PAX6/NESTIN double positive NPCs was significantly higher when the 3D induction method was applied, independent of the genetic background of the cell lines.
- The percentage of SOX1 positive neural progenitor cells was increased using 2D induction.
- The rate of differentiation into neural crest cells by both methods was cell line dependent, which was supported by the rate of the glial differentiation.
- The differentiation rates into cortical subpopulations were not significantly different between the two induction methods.
- Both induction methods supported differentiation into mature, electrophysically active cortical neurons.

The third major question asked here addressed the capacity of neural stem cell potential towards mature astrocytes, aiming to describe the critical steps concerning the generation of astrocytes from embryonic stem cells and induced pluripotent stem cells (Chandrasekaran et al.: Front. Cell. Neuroscience. 10, 215; 2016).

- I have created mouse astrocyte from mouse embryonic stem cells.
- I was able to characterize the *in vitro* generated astrocytes on their phenotypic features (GFAP and AQP4).
- The same protocol was implemented in human NPCs with slight modification. I proved the phenotypic features successfully.

6.5 Future Perspectives

Several key questions remain to be further addressed in future.

1. Effects of GDNF and BDNF on neural stem cells. Although many reports have shown that increased stimulation of BDNF and GDNF during early patterning controls the maturation of neurons. However, ours studies failed to show the effects of chemotaxis. Therefore, it would be interesting to follow up chemotaxis studies in diseased lines.

2. Based on human clinical studies APOE isoform type and level play a role in the development of AD. Currently, the issue whether altering human APOE level affects Aβ pathology remains unclear. Therefore, it is important to address in the future in the *in vitro* models whether it is better to increase or decrease human APOE levels (regardless of isoform) to reduce Aβ levels.

3. I have produced successfully human astrocytes from iPSCs. However, further studies will be needed to characterize the functional features of these cells and the effect of co-culture with these cells on human *in vitro* neuronal models.

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7. ÖSSZEFOGLALÁS

A humán idegi prekurzor sejtek (hNPC) egyik lehetséges felhasználását jelentheti a jövőben az olyan idegrendszeri betegségek kezelése, mint a gerincsérülés vagy a sztrók. Mindemellett hNPC sejtekkel lehetővé válik a genetikai háttér, vagy patogén mutációk hatásának *in vitro* modellezése is. Az elmúlt években hatalmas előrelépés történt számos betegség kórélettanának feltárásában, amely hatóanyag könyvtárak tesztelése alkalmas új sejtes betegségmodellek létrehozását eredményezte. Az idegrendszeri betegségek modellezése egy különösen fontos terület, mivel az emberi központi idegrendszerből *in vivo* minták csak nagyon korlátozottan hozzáférhetőek. Az indukált pluripotens őssejt (iPSC) technológia napjainkban lehetővé tette az olyan idegrendszeri betegségek (pl. Alzheimer kór) tanulmányozását, amelyek vizsgálatához idáig nem rendelkeztünk teljesen megfelelő humán modellrendszerrel. Habár számos, egér és humán neurális sejtdifferenciáltatási protokoll került kidolgozásra, többségük arra alapult, hogy a tenyésztési rendszer hatással van az idegi előalakok *in vitro* funkcionális tulajdonságaira. Azonban a jelentős előrelépések ellenére is, az ún. „3D-alapú aggregációs” és a „2D monolayer” indukciós eljárás és tenyészetek közvetlen, lépésről-lépésre történő összehasonlítása mindeddig váratott magára. A két tenyésztési rendszert ugyanolyan médium, növekedési faktorok és extracelluláris mátrix használata mellett szükséges vizsgálni és összehasonlítani az idegi őssejtek számát, génexpressziós profilját, osztódási és differenciációs képességét, valamint sejtsorsát és funkcionalitását. Munkánk során egy állatmodellt –az egér embrionális őssejteket (mESC) – használtunk, hogy összehasonlítsuk e két neurális differenciációs rendszert. A kapott eredmények alapján, a kidolgozott egér modellrendszer segítségével azt vizsgáltuk, hogy milyen hatással van a genetikai háttér a humán őssejtek neurális irányú elköteleződésére. Ehhez beteg és egészséges donorok sejtjeiből visszaprogramozott humán iPSC sejtek idegi indukcióját végeztük el 2D és 3D rendszer segítségével. Mindkét módszer alkalmazása során ún. neurális rozetták és az azokat alkotó idegi őssejtek (NPC) megjelenését tapasztaltuk, amelyeket az agykérget alkotó sejtekké lehetett továbbtenyészteni. Az eredményeink megmutatták, hogy az alkalmazott protokoll segítségével az NPC sejtek PAX6 expressziója növelhető, és az asztrocita irányú differenciáció elősegíthető. Emellett megmutattuk, hogy a 3D módszerrel előállított NPC sejtekből nagyobb neurit növekedés érhető el. A humán iPSC sejtekből képzett neuronok elektrofiziológiai tulajdonságai hasonlóak az *in vivo* rágcsáló idegsejtekéhez; intenzív feszültségfüggő nátriumcsatorna ionáramlásokkal és akciós potenciál-generáló képességgel rendelkeznek. Mindazonáltal az alkalmazott 2D és 3D módszerek, vagy a különböző genetikai hátterű sejtvonalak között elektrofiziológiai különbségeket nem mutattunk ki. Következtetésképp elmondható, hogy az új modellekkel bepillantást nyerhetünk az emberi idegrendszer korai fejlődési szakaszaiba amely által jobban tanulmányozhatóvá válnak az ehhez kapcsolódó betegségek is. Munkánk során az egér *in vitro* modellrendszer hasznos információkkal szolgált mind a humán agykérgi, mind az asztroglia irányú neurális differenciációs folyamatokkal kapcsolatban. Bizonyítottuk, hogy az NPC sejtek segítségével az embrionális fejlődés egyes szakaszai sikeresen modellezhetőek és így lehetővé válik a fejlődés és elköteleződés során a sejtekben zajló lépések vizsgálata. Emellett, ha ezek a sejtek genetikailag módosított embrionális vagy pluripotens őssejtekből származnak, illetve ha beteg donortól, akkor a továbbiakban az egyes betegségek kórélettana, vagy a hozzá kapcsolódó gyógyszerterápiás lehetőségek is sikerrel vizsgálhatóak.

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8. SUMMARY

Human neural progenitor cells (hNPCs) have the potential to be therapeutically beneficial in the treatment of neuronal diseases such as spinal cord injury or stroke. Recapitulating human neural development *in vitro* using hPSCs can provide us with lot more information on genetic variation, disease-causing mutations and their relevance in affecting neural development. In recent years, tremendous progress has been made in understanding the mechanisms underlying the pathophysiology of certain diseases, which could serve as cell-based disease models for screening chemical libraries. In particular, modeling neurological disease is of great interest, given that it is difficult to obtain patient-derived neural cells because of the limited accessibility of the CNS. iPSC technology now offers us the new possibility to study neural disease, like sporadic Alzheimer's disease, which was not possible on human neuronal model previously.

Although, several groups have independently developed neural differentiation protocols in both mouse and human PSCs, while most of the studies foregrounded the concept that "culture systems" (either 2D or 3D neural induction) can influence the *in vitro* functional properties of neural progenitors. Despite the progress made, no publication directly compared 3D-aggregate and adherent monolayer culture methods using the same growth medium, growth factors or extracellular matrix to evaluate differentiation strategies such as NSC numbers, gene expression profile, proliferation and differentiation potential, cell fate and functionality between the induction methods. In the current study, we used an animal model, the mouse ESCs to compare two well-known neuronal differentiation models. Thereafter, based on the results, we compared these two methods for differentiating different genetic background hiPSCs (healthy and patient-derived ones) into default neural fate through the "mouse modeled" induction systems (2D and 3D neural inductions) to study the neural identity and neural fate of human PSCs. We found that neural rosettes appeared in both the induction methods preferably acquired a cortical identity, which facilitated differentiation into mature cortical neurons. Our results demonstrated that we were able to obtain NPCs with increased PAX6 expression from our NPC pool, resulting promotion of astrocyte differentiation. Additionally, we revealed that by inducing those through 3D induction methods could yield longer neurite length; furthermore, electrophysiology measurements were performed on the generated neural cells. The electrophysiological properties of human iPSCs derived neurons are similar to those of rodent neurons *in vivo*, with large voltage-gated sodium currents and the ability to fire action potentials; however, electrophysiology studies did not reveal a significant difference between the different methods (2D vs3D) or cell lines. Thus, our results demonstrated that we could now provide a novel mechanistic insight into human neural development to study early changes in neural progenitor cells with a particular focus on neurological disease. In this work, an *in vitro* mouse model system proved to be a valid cellular model and was able to provide relevant information to translate the neuronal differentiation protocols, both cortical neuron and astroglial differentiation to the human system. Here, we proved that PSCs could be efficiently used to model embryonic and fetal development and to be used to support developmental and differentiation related decisions. Moreover, if PSCs are derived from engineered models (ESCs or iPSCs of the two species) or diseased patients, they can be used as disease pathology models or drug development tools.

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9. REFERENCES

Abbott, N. J., Rönnbäck, L. and Hansson, E. (2006) 'Astrocyte-endothelial interactions at the blood-brain barrier.', *Nature reviews. Neuroscience*, 7(1), pp. 41–53. doi: 10.1038/nrn1824.

Adewumi, O., Aflatoonian, B., Ahrlund-Richter, L., Amit, M., Andrews, P. W., Beighton, G., Bello, P. A., Benvenisty, N., Berry, L. S., Bevan, S., Blum, B., Brooking, J., Chen, K. G., Choo, A. B. H., Churchill, G. A., Corbel, M., Damjanov, I., Draper, J. S., Dvorak, P., Emanuelsson, K., Fleck, R. A., Ford, A., Gertow, K., Gertsenstein, M., Gokhale, P. J., Hamilton, R. S., Hampl, A., Healy, L. E., Hovatta, O., Hyllner, J., Imreh, M. P., Itskovitz-Eldor, J., Jackson, J., Johnson, J. L., Jones, M., Kee, K., King, B. L., Knowles, B. B., Lako, M., Lebrin, F., Mallon, B. S., Manning, D., Mayshar, Y., McKay, R. D. G., Michalska, A. E., Mikkola, M., Mileikovsky, M., Minger, S. L., Moore, H. D., Mummery, C. L., Nagy, A., Nakatsuji, N., O'Brien, C. M., Oh, S. K. W., Olsson, C., Otonkoski, T., Park, K.-Y., Passier, R., Patel, H., Patel, M., Pedersen, R., Pera, M. F., Piekarczyk, M. S., Pera, R. A. R., Reubinoff, B. E., Robins, A. J., Rossant, J., Rugg-Gunn, P., Schulz, T. C., Semb, H., Sherrer, E. S., Siemen, H., Stacey, G. N., Stojkovic, M., Suemori, H., Szatkiewicz, J., Turetsky, T., Tuuri, T., van den Brink, S., Vintersten, K., Vuoristo, S., Ward, D., Weaver, T. A., Young, L. A. and Zhang, W. (2007) 'Characterization of human embryonic stem cell lines by the International Stem Cell Initiative.', *Nature biotechnology*, 25(7), pp. 803–16. doi: 10.1038/nbt1318.

Agholme, L., Lindström, T., Kågedal, K., Marcusson, J. and Hallbeck, M. (2010) 'An in vitro model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons.', *Journal of Alzheimer's disease : JAD*, 20(4), pp. 1069–82. doi: 10.3233/JAD-2010-091363.

Alcock, J., Lowe, J., England, T., Bath, P. and Sottile, V. (2009) 'Expression of Sox1, Sox2 and Sox9 is maintained in adult human cerebellar cortex', *Neuroscience Letters*, 450(2), pp. 114–116. doi: 10.1016/j.neulet.2008.11.047.

Allegrucci, C. and Young, L. E. (2007) 'Differences between human embryonic stem cell lines', *Human Reproduction Update*, 13(2), pp. 103–120. doi: 10.1093/humupd/dml041.

Andrews, P. W., Matin, M. M., Bahrami, A. R., Damjanov, I., Gokhale, P. and Draper, J. S. (2005) 'Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin', *Biochemical Society Transactions*, 33(6), p. 1526. doi: 10.1042/BST20051526.

Antoni, D., Burckel, H., Josset, E. and Noel, G. (2015) 'Three-dimensional cell culture: a breakthrough in vivo.', *International journal of molecular sciences*. Multidisciplinary Digital Publishing Institute (MDPI), 16(3), pp. 5517–27. doi: 10.3390/ijms16035517.

Araque, A. and Navarrete, M. (2010) 'Glial cells in neuronal network function', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 365(1551), pp. 2375–2381. doi: 10.1098/rstb.2009.0313.

Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N. and Lovell-Badge, R. (2003) 'Multipotent cell lineages in early mouse development depend on SOX2 function', *Genes and Development*, 17(1), pp. 126–140. doi: 10.1101/gad.224503.

Azevedo, F. A. C., Carvalho, L. R. B., Grinberg, L. T., Farfel, J. M., Ferretti, R. E. L., Leite, R. E. P., Filho, W. J., Lent, R. and Herculano-Houzel, S. (2009) 'Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain', *Journal of Comparative Neurology*, 513(5), pp. 532–541. doi: 10.1002/cne.21974.

Baharvand, H., Hashemi, S. M., Kazemi Ashtiani, S. and Farrokhi, A. (2006) 'Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro', *Int J Dev Biol*, 50(7), pp. 645–652. doi: 10.1387/ijdb.052072hb.

Bahmad, H., Hadadeh, O., Chamaa, F., Cheaito, K., Darwish, B., Makkawi, A.-K. and Abou-Kheir, W. (2017) 'Modeling Human Neurological and Neurodegenerative Diseases: From Induced Pluripotent Stem Cells to Neuronal Differentiation and Its Applications in Neurotrauma', *Frontiers in Molecular Neuroscience*, 10. doi: 10.3389/fnmol.2017.00050.

Bal-Price, A. K., Hogberg, H. T., Buzanska, L. and Coecke, S. (2010) 'Relevance of in vitro neurotoxicity testing for regulatory requirements: Challenges to be considered', *Neurotoxicology and Teratology*, 32(1), pp. 36–41. doi: 10.1016/j.ntt.2008.12.003.

Ballen, K. K., Gluckman, E., Broxmeyer, H. E. and DC, W. (2014) 'Umbilical cord blood transplantation : the first 25 years and beyond', *BLOOD*, 122(4), pp. 491–498. doi: 10.1182/blood-2013-02-453175.

Ban, H., Nishishita, N., Fusaki, N., Tabata, T., Saeki, K., Shikamura, M., Takada, N., Inoue, M., Hasegawa, M., Kawamata, S. and Nishikawa, S. (2011) 'Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive Sendai virus vectors', *Proc Natl Acad Sci U S A*, 108(34), pp. 14234–14239. doi: 1103509108 [pii]\r10.1073/pnas.1103509108.

Bélanger, M. and Magistretti, P. J. (2009) 'The role of astroglia in neuroprotection', *Dialogues in Clinical Neuroscience*, pp. 281–296.

Bergsland, M., Ramsköld, D., Zaouter, C., Klum, S., Sandberg, R. and Muhr, J. (2011) 'Sequentially acting Sox transcription factors in neural lineage development', *Genes and Development*, 25(23), pp. 2453–2464. doi: 10.1101/gad.176008.111.

Bergström, T. and Forsberg-Nilsson, K. (2012) 'Neural stem cells: Brain building blocks and beyond', *Upsala Journal of Medical Sciences*, 117(2), pp. 132–142. doi: 10.3109/03009734.2012.665096.

Birgersdotter, A., Sandberg, R. and Ernberg, I. (2005) 'Gene expression perturbation in vitro—A growing case for three-dimensional (3D) culture systems', *Seminars in Cancer Biology*, 15(5), pp. 405–412. doi: 10.1016/j.semcancer.2005.06.009.

Bissonnette, C. J., Lyass, L., Bhattacharyya, B. J., Belmadani, A., Miller, R. J. and Kessler, J. a (2011) 'The controlled generation of functional basal forebrain cholinergic neurons from human embryonic stem cells.', *Stem cells (Dayton, Ohio)*, 29(5), pp. 802–11. doi: 10.1002/stem.626.

Bjorklund, L. M., Sánchez-Pernaute, R., Chung, S., Andersson, T., Chen, I. Y. C., McNaught, K. S. P., Brownell, A.-L., Jenkins, B. G., Wahlestedt, C., Kim, K.-S. and Isacson, O. (2002) 'Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model.', *Proceedings of the National Academy of Sciences of the United States of America*, 99(4), pp. 2344–9. doi: 10.1073/pnas.022438099.

Blackshaw, S. E., Arkison, S., Cameron, C. and Davies, J. a (1997) 'Promotion of regeneration and axon growth following injury in an invertebrate nervous system by the use of three-dimensional collagen gels.', *Proceedings. Biological sciences / The Royal Society*, 264(1382), pp. 657–661. doi: 10.1098/rspb.1997.0093.

Boheler, K. R., Czyz, J., Tweedie, D., Yang, H. T., Anisimov, S. V. and Wobus, A. M. (2002) 'Differentiation of pluripotent embryonic stem cells into cardiomyocytes', *Circulation Research*, pp. 189–201. doi: 10.1161/01.RES.0000027865.61704.32.

Bouet, G., Marchat, D., Cruel, M., Malaval, L. and Vico, L. (2015) 'In vitro three-dimensional bone tissue models: from cells to controlled and dynamic environment.', *Tissue engineering. Part B, Reviews*, 21(1), pp. 133–56. doi: 10.1089/ten.TEB.2013.0682.

Brack, A. S. and Rando, T. A. (2012) 'Tissue-specific stem cells: lessons from the skeletal muscle satellite cell.', *Cell stem cell*. NIH Public Access, 10(5), pp. 504–14. doi:

10.1016/j.stem.2012.04.001.

Brenner, M., Goldman, J. E., Quinlan, R. A., Messing, A., Parpura, V., Haydon, P. G. and Brenner, M. (2009) ‘Alexander Disease: A Genetic Disorder of Astrocytes’, *Springer*, 11(1), p. pp 591-648. doi: 10.1007/978-0-387-79492-1_24.

Brown, R. C., Mark, K. S., Egleton, R. D., Huber, J. D., Burroughs, A. R. and Davis, T. P. (2003) ‘Protection against hypoxia-induced increase in blood-brain barrier permeability: role of tight junction proteins and NFkappaB.’, *Journal of cell science*, 116(Pt 4), pp. 693–700. doi: 10.1242/jcs.00264.

Buehr, M., Meek, S., Blair, K., Yang, J., Ure, J., Silva, J., McLay, R., Hall, J., Ying, Q. L. and Smith, A. (2008) ‘Capture of Authentic Embryonic Stem Cells from Rat Blastocysts’, *Cell*, 135(7), pp. 1287–1298. doi: 10.1016/j.cell.2008.12.007.

Buganim, Y., Faddah, D. A. and Jaenisch, R. (2013) ‘Mechanisms and models of somatic cell reprogramming.’, *Nature reviews. Genetics*, 14(6), pp. 427–39. doi: 10.1038/nrg3473.

Butterworth, R. F. (2010) ‘Altered glial–neuronal crosstalk: Cornerstone in the pathogenesis of hepatic encephalopathy’, *Neurochemistry International*, 57(4), pp. 383–388. doi: 10.1016/j.neuint.2010.03.012.

Cai, J., Schleidt, S., Pelta-Heller, J., Hutchings, D., Cannarsa, G. and Iacovitti, L. (2013) ‘BMP and TGF-β pathway mediators are critical upstream regulators of Wnt signaling during midbrain dopamine differentiation in human pluripotent stem cells’, *Developmental Biology*, 376(1), pp. 62–73. doi: 10.1016/j.ydbio.2013.01.012.

Campbell, K. H., McWhir, J., Ritchie, W. A. and Wilmut, I. (1996) ‘Sheep cloned by nuclear transfer from a cultured cell line.’, *Nature*, pp. 64–66. doi: 10.1038/380064a0.

Carlén, M., Meletis, K., Göritz, C., Darsalia, V., Evergren, E., Tanigaki, K., Amendola, M., Barnabé-Heider, F., Yeung, M. S. Y., Naldini, L., Honjo, T., Kokaia, Z., Shupliakov, O., Cassidy, R. M., Lindvall, O. and Frisén, J. (2009) ‘Forebrain ependymal cells are Notch-dependent and generate neuroblasts and astrocytes after stroke.’, *Nature neuroscience*, 12(3), pp. 259–267. doi: 10.1038/nn.2268.

Carneiro, M., Afonso, S., Geraldes, A., Garreau, H., Bolet, G., Boucher, S., Tircazes, A., Queney, G., Nachman, M. W. and Ferrand, N. (2011) ‘The Genetic Structure of Domestic Rabbits’, *Molecular Biology and Evolution*. Oxford University Press, 28(6), pp. 1801–1816. doi: 10.1093/molbev/msr003.

Cartwright, P., McLean, C., Sheppard, A., Rivett, D., Jones, K. and Dalton, S. (2005) ‘LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism’, *Development*, 132(5), pp. 885–896. doi: 10.1242/dev.01670.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. and Smith, A. (2003) ‘Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells.’, *Cell*, 113(5), pp. 643–55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12787505 (Accessed: 29 March 2017).

Chambers, S. M., Fasano, C. A., Papapetrou, E. P., Tomishima, M., Sadelain, M. and Studer, L. (2009) ‘Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling.’, *Nature biotechnology*, 27(3), pp. 275–80. doi: 10.1038/nbt.1529.

Chandrasekaran, A., Varga, E., Nemes, C., Táncos, Z., Kobolák, J. and Dinnyés, A. (2016) ‘Establishment of induced pluripotent stem cell (iPSC) line from a 63-year old patient with late onset Alzheimer’s disease (LOAD)’, *Stem Cell Research*, 17(1), pp. 78–80. doi: 10.1016/j.scr.2016.05.014.

Chaudry, A. S. (2004) *Stem Cell Bioengineering*. Available at: http://www.scq.ubc.ca/stem-cell-bioengineering/.

Chen, H., Qian, K., Du, Z., Cao, J., Petersen, A., Liu, H., Blackbourn, L. W., Huang, C.-L., Errigo, A., Yin, Y., Lu, J., Ayala, M. and Zhang, S.-C. (2014) 'Modeling ALS with iPSCs reveals that mutant SOD1 misregulates neurofilament balance in motor neurons.', *Cell stem cell*, 14(6), pp. 796–809. doi: 10.1016/j.stem.2014.02.004.

Chen, H., Qian, K., Du, Z., Cao, J., Petersen, A., Liu, H., Blackbourn, L. W., Huang, C. L., Errigo, A., Yin, Y., Lu, J., Ayala, M. and Zhang, S. C. (2014) 'Modeling ALS with iPSCs reveals that mutant SOD1 misregulates neurofilament balance in motor neurons', *Cell Stem Cell*, 14(6), pp. 796–809. doi: 10.1016/j.stem.2014.02.004.

Chen, Y.-F., Tseng, C.-Y., Wang, H.-W., Kuo, H.-C., Yang, V. W. and Lee, O. K. (2012) 'Rapid generation of mature hepatocyte-like cells from human induced pluripotent stem cells by an efficient three-step protocol.', *Hepatology (Baltimore, Md.)*, 55(4), pp. 1193–203. doi: 10.1002/hep.24790.

Chesselet, M.-F. and Carmichael, S. T. (2012) 'Animal models of neurological disorders.', *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics*. Springer, 9(2), pp. 241–4. doi: 10.1007/s13311-012-0118-9.

Chou, S., Chu, P., Hwang, W. and Lodish, H. (2010) 'Expansion of human cord blood hematopoietic stem cells for transplantation', *Cell Stem Cell*, 7(4), pp. 427–428. doi: 10.1016/j.stem.2010.09.001.

Clarke, L. E. and Barres, B. a (2013) 'Emerging roles of astrocytes in neural circuit development.', *Nature reviews. Neuroscience*, 14(5), pp. 311–21. doi: 10.1038/nrn3484.

Cockburn, K. and Rossant, J. (2010) 'Making the blastocyst: Lessons from the mouse', *Journal of Clinical Investigation*, pp. 995–1003. doi: 10.1172/JCI41229.

Corti, S., Faravelli, I., Cardano, M. and Conti, L. (2015) 'Human pluripotent stem cells as tools for neurodegenerative and neurodevelopmental disease modeling and drug discovery', *Expert Opinion on Drug Discovery*, 10(6), pp. 615–629. doi: 10.1517/17460441.2015.1037737.

Coulter, D. A. and Eid, T. (2012) 'Astrocytic regulation of glutamate homeostasis in epilepsy', *Glia*, 60(8), pp. 1215–1226. doi: 10.1002/glia.22341.

Czechanski, A., Byers, C., Greenstein, I., Schrode, N., Donahue, L. R., Hadjantonakis, A.-K. and Reinholdt, L. G. (2014) 'Derivation and characterization of mouse embryonic stem cells from permissive and nonpermissive strains.', *Nature protocols*, 9(3), pp. 559–74. doi: 10.1038/nprot.2014.030.

Czepiel, M., Balasubramaniyan, V., Schaafsma, W., Stancic, M., Mikkers, H., Huisman, C., Boddeke, E. and Copray, S. (2011) 'Differentiation of induced pluripotent stem cells into functional oligodendrocytes', *Glia*, 59(6), pp. 882–892. doi: 10.1002/glia.21159.

Danbolt, N. C. (2001) 'Glutamate uptake', *Progress in Neurobiology*, 65(1), pp. 1–105. doi: 10.1016/S0301-0082(00)00067-8.

Dash, B. C., Jiang, Z., Suh, C. and Qyang, Y. (2015) 'Induced pluripotent stem cell-derived vascular smooth muscle cells: methods and application.', *The Biochemical journal*, 465(2), pp. 185–94. doi: 10.1042/BJ20141078.

Davidson, K. C., Mason, E. A. and Pera, M. F. (2015) 'The pluripotent state in mouse and human', *Development*, 142(18).

Devine, M., Ryten, M., Vodicka, P., Thomson, A., Burdon, T., Houlden, H., Cavaleri, F., Nagano, M., Drummond, N., Taanman, J.-W., Schapira, A., Gwinn, K., Hardy, J., Lewis, P. and

Kunath, T. (2011) 'Parkinson's disease induced pluripotent stem cells with triplication of the α-synuclein locus', *Nature Communications*, 2(1), p. 440. doi: 10.1038/ncomms1453.

Dhara, S. K. and Stice, S. L. (2008) 'Neural differentiation of human embryonic stem cells', *Journal of Cellular Biochemistry*, pp. 633–640. doi: 10.1002/jcb.21891.

Diniz, L. P., Almeida, J. C., Tortelli, V., Lopes, C. V., Setti-Perdigão, P., Stipursky, J., Kahn, S. A., Romão, L. F., De Miranda, J., Alves-Leon, S. V., De Souza, J. M., Castro, N. G., Panizzutti, R. and Gomes, F. C. A. (2012) 'Astrocyte-induced synaptogenesis is mediated by transforming growth factor β signaling through modulation of d-serine levels in cerebral cortex neurons', *Journal of Biological Chemistry*, 287(49), pp. 41432–41445. doi: 10.1074/jbc.M112.380824.

Dinnyés, A., De Sousa, P., King, T. and Wilmut, I. (2002) 'Somatic cell nuclear transfer: recent progress and challenges.', *Cloning and stem cells*, 4(1), pp. 81–90. doi: 10.1089/153623002753632075.

Doke, S. K. and Dhawale, S. C. (2015) 'Alternatives to animal testing: A review.', *Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society*, 23(3), pp. 223–9. doi: 10.1016/j.jsps.2013.11.002.

Dowey, S. N., Huang, X., Chou, B.-K., Ye, Z. and Cheng, L. (2012) 'Generation of integration-free human induced pluripotent stem cells from postnatal blood mononuclear cells by plasmid vector expression.', *Nature protocols*, 7(11), pp. 2013–21. doi: 10.1038/nprot.2012.121.

Du, Z.-W., Chen, H., Liu, H., Lu, J., Qian, K., Huang, C.-L., Zhong, X., Fan, F. and Zhang, S.-C. (2015) 'Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells', *Nature Communications*. Nature Publishing Group, 6, p. 6626. doi: 10.1038/ncomms7626.

Eakin, G. S. and Hadjantonakis, A.-K. (2006) 'Production of chimeras by aggregation of embryonic stem cells with diploid or tetraploid mouse embryos.', *Nature protocols*. NIH Public Access, 1(3), pp. 1145–53. doi: 10.1038/nprot.2006.173.

Emdad, L., D'Souza, S. L., Kothari, H. P., Qadeer, Z. A. and Germano, I. M. (2012) 'Efficient Differentiation of Human Embryonic and Induced Pluripotent Stem Cells into Functional Astrocytes', *Stem Cells and Development*, 21(3), pp. 404–410. doi: 10.1089/scd.2010.0560.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996) 'Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity', *Cell*, 87(4), pp. 661–673. doi: S0092-8674(00)81386-0 [pii].

Espuny-Camacho, I., Michelsen, K. A., Gall, D., Linaro, D., Hasche, A., Bonnefont, J., Bali, C., Orduz, D., Bilheu, A., Herpoel, A., Lambert, N., Gaspard, N., Péron, S., Schiffmann, S. N., Giugliano, M., Gaillard, A. and Vanderhaeghen, P. (2013) 'Pyramidal Neurons Derived from Human Pluripotent Stem Cells Integrate Efficiently into Mouse Brain Circuits In Vivo', *Neuron*, 77(3), pp. 440–456. doi: 10.1016/j.neuron.2012.12.011.

Esteban, M. A., Xu, J., Yang, J., Peng, M., Qin, D., Li, W., Jiang, Z., Chen, J., Deng, K., Zhong, M., Cai, J., Lai, L. and Pei, D. (2009) 'Generation of induced pluripotent stem cell lines from Tibetan miniature pig', *Journal of Biological Chemistry*, 284(26), pp. 17634–17640. doi: 10.1074/jbc.M109.008938.

Evans, M. J. and Kaufman, M. H. (1981) 'Establishment in culture of pluripotential cells from mouse embryos', *Nature*. Nature Publishing Group, 292(5819), pp. 154–156. doi: 10.1038/292154a0.

Evans, M. J. and Kaufman, M. H. (1981) 'Establishment in culture of pluripotential cells from mouse embryos.', *Nature*, pp. 154–156. doi: 10.1038/292154a0.

Faravelli, I., Bucchia, M., Rinchetti, P., Nizzardo, M., Simone, C., Frattini, E. and Corti, S. (2014) 'Motor neuron derivation from human embryonic and induced pluripotent stem cells: experimental approaches and clinical perspectives', *Stem Cell Research & Therapy*, 5(4), p. 87. doi: 10.1186/scrt476.

Fathi, A., Rasouli, H., Yeganeh, M., Salekdeh, G. H. and Baharvand, H. (2015) 'Efficient Differentiation of Human Embryonic Stem Cells Toward Dopaminergic Neurons Using Recombinant LMX1A Factor', *Molecular Biotechnology*, 57(2), pp. 184–194. doi: 10.1007/s12033-014-9814-5.

Felipo, V. and Butterworth, R. F. (2002) 'Neurobiology of ammonia', *Progress in Neurobiology*, 67(4), pp. 259–279. doi: 10.1016/S0301-0082(02)00019-9.

Forraz, N. and Mcguckin, C. P. (2011) 'The umbilical cord: A rich and ethical stem cell source to advance regenerative medicine', *Cell Proliferation*, 44(SUPPL. 1), pp. 60–69. doi: 10.1111/j.1365-2184.2010.00729.x.

Franke, H. and Kittner, H. (2001) 'Morphological alterations of neurons and astrocytes and changes in emotional behavior in pentylenetetrazol-kindled rats', *Pharmacology Biochemistry and Behavior*, 70(2–3), pp. 291–303. doi: 10.1016/S0091-3057(01)00612-8.

Freeman, M. R. (2010) 'Specification and morphogenesis of astrocytes.', *Science (New York, N.Y.)*, 330(6005), pp. 774–778. doi: 10.1126/science.1190928.

Frimat, J.-P., Xie, S., Bastiaens, A., Schurink, B., Wolbers, F., den Toonder, J. and Luttge, R. (2015) 'Advances in 3D neuronal cell culture', *Journal of Vacuum Science & Technology B, Nanotechnology and Microelectronics: Materials, Processing, Measurement, and Phenomena*, 33(6), p. 06F902. doi: 10.1116/1.4931636.

Fusaki, N., Ban, H., Nishiyama, A., Saeki, K. and Hasegawa, M. (2009) 'Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome.', *Proceedings of the Japan Academy. Series B, Physical and biological sciences*. The Japan Academy, 85(8), pp. 348–62. doi: 10.2183/pjab.85.348.

Faas, L., Warrander, F. C., Maguire, R., Ramsbottom, S. a, Quinn, D., Genever, P. and Isaacs, H. V (2013) 'Lin28 proteins are required for germ layer specification in Xenopus.', *Development (Cambridge, England)*, 140(5), pp. 976–86. doi: 10.1242/dev.089797.

Gafni, O., Weinberger, L., Mansour, A. A., Manor, Y. S., Chomsky, E., Ben-Yosef, D., Kalma, Y., Viukov, S., Maza, I., Zviran, A., Rais, Y., Shipony, Z., Mukamel, Z., Krupalnik, V., Zerbib, M., Geula, S., Caspi, I., Schneir, D., Shwartz, T., Gilad, S., Amann-Zalcenstein, D., Benjamin, S., Amit, I., Tanay, A., Massarwa, R., Novershtern, N. and Hanna, J. H. (2013) 'Derivation of novel human ground state naive pluripotent stem cells', *Nature*, 504(7479), pp. 282–286. doi: 10.1038/nature12745.

Gámez, B., Rodriguez-Carballo, E. and Ventura, F. (2013) 'BMP signaling in telencephalic neural cell specification and maturation.', *Frontiers in cellular neuroscience*, 7(June), p. 87. doi: 10.3389/fncel.2013.00087.

Gammill, L. S. and Bronner-Fraser, M. (2003) 'Neural crest specification: migrating into genomics.', *Nature reviews. Neuroscience*, 4(10), pp. 795–805. doi: 10.1038/nrn1219.

Geckil, H., Xu, F., Zhang, X., Moon, S. and Demirci, U. (2010) 'Engineering hydrogels as extracellular matrix mimics.', *Nanomedicine (London, England)*, 5(3), pp. 469–84. doi: 10.2217/nnm.10.12.

Gerrard, L., Rodgers, L. and Cui, W. (2005) 'Differentiation of human embryonic stem cells to neural lineages in adherent culture by blocking bone morphogenetic protein signaling', *Stem*

Cells, 23(1066–5099 (Print)), pp. 1234–1241. doi: 10.1634/stemcells.2005-0110.

Ginis, I., Luo, Y., Miura, T., Thies, S., Brandenberger, R., Gerecht-Nir, S., Amit, M., Hoke, A., Carpenter, M. K., Itskovitz-Eldor, J. and Rao, M. S. (2004) 'Differences between human and mouse embryonic stem cells', *Dev Biol*, 269(2), pp. 360–380. doi: 10.1016/j.ydbio.2003.12.034\rS0012160604000193 [pii].

Gitler, A., Dhillon, P. and Shorter, J. (2017) 'Neurodegenerative disease: models, mechanisms, and a new hope', *The Company of Biologists*, 10(1), pp. 499–502. doi: 10.1242/dmm.030205.

Gont, L. K., Steinbeisser, H., Blumberg, B. and de Robertis, E. M. (1993) 'Tail formation as a continuation of gastrulation: the multiple cell populations of the Xenopus tailbud derive from the late blastopore lip.', *Development (Cambridge, England)*, 119(4), pp. 991–1004. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7916680 (Accessed: 23 September 2016).

Gorris, R., Fischer, J., Erwes, K. L., Kesavan, J., Peterson, D. A., Alexander, M., Nöthen, M. M., Peitz, M., Quandel, T., Karus, M. and Brüstle, O. (2015) 'Pluripotent stem cell-derived radial glia-like cells as stable intermediate for efficient generation of human oligodendrocytes', *GLIA*, 63(12), pp. 2152–2167. doi: 10.1002/glia.22882.

Greene, N. D. E. and Copp, A. J. (2009) 'Development of the vertebrate central nervous system: Formation of the neural tube', *Prenatal Diagnosis*, pp. 303–311. doi: 10.1002/pd.2206.

Guillot, P. V., O'Donoghue, K., Kurata, H. and Fisk, N. M. (2006) 'Fetal stem cells: Betwixt and between', *Seminars in Reproductive Medicine*, pp. 340–347. doi: 10.1055/s-2006-952149.

Gurdon, J. B., Lane, C. D., Woodland, H. R. and Marbaix, G. (1971) 'Use of Frog Eggs and Oocytes for the Study of Messenger RNA and its Translation in Living Cells', *Nature*. Nature Publishing Group, 233(5316), pp. 177–182. doi: 10.1038/233177a0.

Gurdon, J. B., Laskey, R. a and Reeves, O. R. (1975) 'The developmental capacity of nuclei transplanted from keratinized skin cells of adult frogs.', *Journal of embryology and experimental morphology*, 34(1), pp. 93–112.

Haeckel, E., Fol, H. and Strasburger (1850) 'Natürliche Schöpfungsgeschichte', pp. 1850–1924.

Hall, V. J. (2016) 'Modelling Neurodegenerative Diseases Using Human Pluripotent Stem Cells', in *Pluripotent Stem Cells - From the Bench to the Clinic*. InTech. doi: 10.5772/63521.

Han, X., Han, J., Ding, F., Cao, S., Lim, S. S., Dai, Y., Zhang, R., Zhang, Y., Lim, B. and Li, N. (2011) 'Generation of induced pluripotent stem cells from bovine embryonic fibroblast cells', *Cell Research*. Nature Publishing Group, 21(10), pp. 1509–1512. doi: 10.1038/cr.2011.125.

Hanna, J., Cheng, A. W., Saha, K., Kim, J., Lengner, C. J., Soldner, F., Cassady, J. P., Muffat, J., Carey, B. W. and Jaenisch, R. (2010) 'Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs.', *Proceedings of the National Academy of Sciences of the United States of America*, 107(20), pp. 9222–9227. doi: 10.1073/pnas.1004584107.

Hata, M., Yamashiro, K., Oishi, A., Ooto, S., Tamura, H., Miyata, M., Ueda-Arakawa, N., Kuroda, Y., Takahashi, A., Tsujikawa, A. and Yoshimura, N. (2017) 'Retinal Pigment Epithelial Atrophy After Anti-Vascular Endothelial Growth Factor Injections For Retinal Angiomatous Proliferation', *Retina*. doi: 10.1097/IAE.0000000000001457.

Henle, G. and Deinhardt, F. (1957) 'The establishment of strains of human cells in tissue culture.', *Journal of immunology*. American Association of Immunologists, 79(1), pp. 54–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13475808.

Hong, Y. K. and Chen, C. (2011) 'Wiring and rewiring of the retinogeniculate synapse.', *Current opinion in neurobiology*, 21(2), pp. 228–37. doi: 10.1016/j.conb.2011.02.007.

Hosseinkhani, H., Hiraoka, Y., Li, C. H., Chen, Y. R., Yu, D. S., Hong, P. Da and Ou, K. L. (2013) 'Engineering three-dimensional collagen-IKVAV matrix to mimic neural microenvironment', *ACS Chemical Neuroscience*, 4(8), pp. 1229–1235. doi: 10.1021/cn400075h.

Hossini, A. M., Megges, M., Prigione, A., Lichtner, B., Toliat, M. R., Wruck, W., Schröter, F., Nuernberg, P., Kroll, H., Makrantonaki, E., Zoubouliss, C. C. and Adjaye, J. (2015) 'Induced pluripotent stem cell-derived neuronal cells from a sporadic Alzheimer's disease donor as a model for investigating AD-associated gene regulatory networks. TL - 16', *BMC genomics*, 16 VN-r, p. 84. doi: 10.1186/s12864-015-1262-5.

Hou, P., Li, Y., Zhang, X., Liu, C., Guan, J., Li, H., Zhao, T., Ye, J., Yang, W., Liu, K., Ge, J., Xu, J., Zhang, Q., Zhao, Y. and Deng, H. (2013) 'Pluripotent Stem Cells Induced from Mouse Somatic Cells by Small-Molecule Compounds', *Science*, 341(6146), pp. 651–654. doi: 10.1126/science.1239278.

Huang, K., Maruyama, T. and Fan, G. (2014) 'The naive state of human pluripotent stem cells: A synthesis of stem cell and preimplantation embryo transcriptome analyses', *Cell Stem Cell*, 15(4), pp. 410–415. doi: 10.1016/j.stem.2014.09.014.

Huangfu, D., Maehr, R., Guo, W., Eijkelenboom, A., Snitow, M., Chen, A. E. and Melton, D. a (2008) 'Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds.', *Nature biotechnology*, 26(7), pp. 795–7. doi: 10.1038/nbt1418.

Hyun, I., Hochedlinger, K., Jaenisch, R. and Yamanaka, S. (2007) 'New Advances in iPS Cell Research Do Not Obviate the Need for Human Embryonic Stem Cells', *Cell Stem Cell*, pp. 367–368. doi: 10.1016/j.stem.2007.09.006.

Imaizumi, Y., Okada, Y., Akamatsu, W., Koike, M., Kuzumaki, N., Hayakawa, H., Nihira, T., Kobayashi, T., Ohyama, M., Sato, S., Takanashi, M., Funayama, M., Hirayama, A., Soga, T., Hishiki, T., Suematsu, M., Yagi, T., Ito, D., Kosakai, A., Hayashi, K., Shouji, M., Nakanishi, A., Suzuki, N., Mizuno, Y., Mizushima, N., Amagai, M., Uchiyama, Y., Mochizuki, H., Hattori, N. and Okano, H. (2012) 'Mitochondrial dysfunction associated with increased oxidative stress and alpha-synuclein accumulation in PARK2 iPSC-derived neurons and postmortem brain tissue', *Molecular Brain*, 5(1), p. 35. doi: 10.1186/1756-6606-5-35.

Israel, M., Yuan, S. H., Bardy, C., Reyna, S. M. S., Mu, Y., Herrera, C., Hefferan, M. P., Van Gorp, S., Nazor, K. L., Boscolo, F. S., Carson, C. T., Laurent, L. C., Marsala, M., Gage, F. H., Remes, A. M., Koo, E. H. and Goldstein, L. S. B. (2012) 'Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells.', *Nature*, 482(7384), pp. 216–20. doi: 10.1038/nature10821.

Jaenisch, R. and Young, R. (2008) 'Stem Cells, the Molecular Circuitry of Pluripotency and Nuclear Reprogramming', *Cell*, pp. 567–582. doi: 10.1016/j.cell.2008.01.015.

Jankovic, J. (2008) 'Parkinson's disease: clinical features and diagnosis', *Journal of Neurology, Neurosurgery & Psychiatry*, 79(4), pp. 368–376. doi: 10.1136/jnnp.2007.131045.

Jha, B. S., Rao, M. and Malik, N. (2015) 'Motor Neuron Differentiation from Pluripotent Stem Cells and Other Intermediate Proliferative Precursors that can be Discriminated by Lineage Specific Reporters', *Stem Cell Reviews and Reports*, 11, pp. 194–204. doi: 10.1007/s12015-014-9541-0.

Jincho, Y., Araki, R., Hoki, Y., Tamura, C., Nakamura, M., Ando, S., Kasama, Y. and Abe, M. (2010) 'Generation of genome integration-free induced pluripotent stem cells from fibroblasts of C57BL/6 mice without c-Myc transduction.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 285(34), pp. 26384–9. doi: 10.1074/jbc.M110.115915.

Johnson, M. A., Weick, J. P., Pearce, R. A. and Zhang, S.-C. (2007) 'Functional Neural Development from Human Embryonic Stem Cells: Accelerated Synaptic Activity via Astrocyte Coculture', *Journal of Neuroscience*, 27(12), pp. 3069–3077. doi: 10.1523/JNEUROSCI.4562-06.2007.

Johnstone, M., Thomson, P. A., Hall, J., McIntosh, A. M., Lawrie, S. M. and Porteous, D. J. (2011) 'DISC1 in Schizophrenia: Genetic Mouse Models and Human Genomic Imaging', *Schizophrenia Bulletin*, 37(1), pp. 14–20. doi: 10.1093/schbul/sbq135.

K.Szasz, B., Mike, A., Karoly, R., Gerevich, Z., Illes, P., Vizi, S. and P.Kiss, J. (2007) 'Direct Inhibitory Effect of Fluoxetine on N-Methyl-D-Aspartate Receptors in the Central Nervous System', *Biological Psychiatry*, 62(11), pp. 1303–9. doi: 10.1016/j.biopsych.2007.04.014.

Kandasamy, M., Lehner, B., Kraus, S., Sander, P. R., Marschallinger, J., Rivera, F. J., Trümbach, D., Ueberham, U., Reitsamer, H. A., Strauss, O., Bogdahn, U., Couillard-Despres, S. and Aigner, L. (2014) 'TGF-beta signalling in the adult neurogenic niche promotes stem cell quiescence as well as generation of new neurons', *Journal of Cellular and Molecular Medicine*, 18(7), pp. 1444–1459. doi: 10.1111/jcmm.12298.

Kang, X., Yu, Q., Huang, Y., Song, B., Chen, Y., Gao, X., He, W., Sun, X., Fan, Y., Zhao, X., Li, W., Lv, Z., Liu, L., Tong, M., Hai, T., Kang, L., Wang, J., Zhang, Y., Kou, Z., Gao, S., Boland, M., Hazen, J., Nazor, K., Rodriguez, A., Gifford, W., Martin, G., Martins-Taylor, K., Nisler, B., Taapken, S., Compton, T., Crandall, L., Laurent, L., Ulitsky, I., Slavin, I., Tran, H., Schork, A., Montgomery, K., Lister, R., Pelizzola, M., Kida, Y., Hawkins, R., Nery, J., Hon, G., Hussein, S., Batada, N., Vuoristo, S., Ching, R., Autio, R., Närvä, E., Goh, P., Caxaria, S., Casper, C., Rosales, C., Warner, T., Coffey, P., Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Warren, L., Manos, P., Ahfeldt, T., Loh, Y., Li, H., Lau, F., Yu, J., Chau, K., Vodyanik, M., Jiang, J., Jiang, Y., Hou, P., Li, Y., Zhang, X., Liu, C., Guan, J., Li, H., Schlaeger, T., Daheron, L., Brickler, T., Entwisle, S., Chan, K., Cianci, A., Chou, B., Mali, P., Huang, X., Ye, Z., Dowey, S., Resar, L., Gore, A., Li, Z., Fung, H., Young, J., Agarwal, S., Antosiewicz-Bourget, J., Fan, Y., Luo, Y., Chen, X., Sun, X., He, W., Kang, X., Du, H., Song, B., Lu, Z., Huang, Y., Fan, Y., Luo, Y., Chen, X., Li, Q., Sun, X., Luo, Y., Li, J., Zhu, D., Fan, Y., Li, S., Sun, X., Bock, C., Kiskinis, E., Verstappen, G., Gu, H., Boulting, G., Smith, Z., Park, T., Huo, J., Peters, A., Jr., C. T., Verma, K., Zimmerlin, L., Macarthur, C., Fontes, A., Ravinder, N., Kuninger, D., Kaur, J., Bailey, M., Okita, K., Yamakawa, T., Matsumura, Y., Sato, Y., Amano, N., Watanabe, A., Newman, A., Cooper, J., Guenther, M., Frampton, G., Soldner, F., Hockemeyer, D., Mitalipova, M., Jaenisch, R., Agarwal, S., Daley, G., Asensi, K., Fortunato, R., Santos, D. dos, Pacheco, T., Rezende, D. de and Rodrigues, D. (2015) 'Effects of Integrating and Non-Integrating Reprogramming Methods on Copy Number Variation and Genomic Stability of Human Induced Pluripotent Stem Cells', *PLOS ONE*. Edited by R. Johnson. Public Library of Science, 10(7), p. e0131128. doi: 10.1371/journal.pone.0131128.

Kaufman, M. H., Robertson, E. J., Handyside, A. H. and Evans, M. J. (1983) 'Establishment of pluripotential cell lines from haploid mouse embryos.', *Journal of embryology and experimental morphology*, 73(1), pp. 249–61. doi: dev.biologists.org/content/73/1/249.long.

Kazutoshi Takahashi and Shinya Yamanaka (2016) 'A decade of transcription factor-mediated reprogramming to pluripotency', *Nature Reviews Molecular Cell Biology*, 17(3), pp. 183–93. doi: 10.1038/nrm.2016.8.

Kehler, J., Tolkunova, E., Koschorz, B., Pesce, M., Gentile, L., Boiani, M., Lomelí, H., Nagy, A., McLaughlin, K. J., Schöler, H. R. and Tomilin, A. (2004) 'Oct4 is required for primordial germ cell survival.', *EMBO reports*, 5(11), pp. 1078–1083. doi: 10.1038/sj.embor.7400279.

Kielman, M. F., Rindapää, M., Gaspar, C., van Poppel, N., Breukel, C., van Leeuwen, S., Taketo, M. M., Roberts, S., Smits, R. and Fodde, R. (2002) 'Apc modulates embryonic stem-cell

differentiation by controlling the dosage of beta-catenin signaling.', *Nature genetics*, 32(4), pp. 594–605. doi: 10.1038/ng1045.

Kiernan, M. C., Vucic, S., Cheah, B. C., Turner, M. R., Eisen, A., Hardiman, O., Burrell, J. R. and Zoing, M. C. (2011) 'Amyotrophic lateral sclerosis.', *Lancet*, 377(9769), pp. 942–955. doi: 10.1016/S0140-6736(10)61156-7.

Kim, D., Kim, C.-H., Moon, J.-I., Chung, Y.-G., Chang, M.-Y., Han, B.-S., Ko, S., Yang, E., Cha, K. Y., Lanza, R. and Kim, K.-S. (2009) 'Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins.', *Cell stem cell*, 4(6), pp. 472–476. doi: 10.1016/j.stem.2009.05.005.

Kirkeby, A., Grealish, S., Wolf, D. A., Nelander, J., Wood, J., Lundblad, M., Lindvall, O. and Parmar, M. (2012) 'Generation of Regionally Specified Neural Progenitors and Functional Neurons from Human Embryonic Stem Cells under Defined Conditions', *Cell Reports*, 1(6), pp. 703–714. doi: 10.1016/j.celrep.2012.04.009.

Kirsch-Volders, M., Plas, G., Elhajouji, A., Lukamowicz, M., Gonzalez, L., Vande Loock, K. and Decordier, I. (2011) 'The in vitro MN assay in 2011: origin and fate, biological significance, protocols, high throughput methodologies and toxicological relevance', *Archives of Toxicology*, 85(8), pp. 873–899. doi: 10.1007/s00204-011-0691-4.

Kiskinis, E., Sandoe, J., Williams, L. a, Boulting, G. L., Moccia, R., Wainger, B. J., Han, S., Peng, T., Thams, S., Mikkilineni, S., Mellin, C., Merkle, F. T., Davis-Dusenbery, B. N., Ziller, M., Oakley, D., Ichida, J., Di Costanzo, S., Atwater, N., Maeder, M. L., Goodwin, M. J., Nemesh, J., Handsaker, R. E., Paull, D., Noggle, S., McCarroll, S. a, Joung, J. K., Woolf, C. J., Brown, R. H. and Eggan, K. (2014) 'Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SOD1.', *Cell stem cell*, 14(6), pp. 781–95. doi: 10.1016/j.stem.2014.03.004.

Kleiderman, S., Sá, J. V., Teixeira, A. P., Brito, C., Gutbier, S., Evje, L. G., Hadera, M. G., Glaab, E., Henry, M., Sachinidis, A., Alves, P. M., Sonnewald, U. and Leist, M. (2016) 'Functional and phenotypic differences of pure populations of stem cell-derived astrocytes and neuronal precursor cells', *GLIA*, pp. 695–715. doi: 10.1002/glia.22954.

Koh, H. S., Yong, T., Chan, C. K. and Ramakrishna, S. (2008) 'Enhancement of neurite outgrowth using nano-structured scaffolds coupled with laminin', *Biomaterials*, 29(26), pp. 3574–3582. doi: 10.1016/j.biomaterials.2008.05.014.

Kondo, M. (2010) 'Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors', *Immunological Reviews*, 238(1), pp. 37–46. doi: 10.1111/j.1600-065X.2010.00963.x.

Kondo, T., Asai, M., Tsukita, K., Kutoku, Y., Ohsawa, Y., Sunada, Y., Imamura, K., Egawa, N., Yahata, N., Okita, K., Takahashi, K., Asaka, I., Aoi, T., Watanabe, A., Watanabe, K., Kadoya, C., Nakano, R., Watanabe, D., Maruyama, K., Hori, O., Hibino, S., Choshi, T., Nakahata, T., Hioki, H., Kaneko, T., Naitoh, M., Yoshikawa, K., Yamawaki, S., Suzuki, S., Hata, R., Ueno, S., Seki, T., Kobayashi, K., Toda, T., Murakami, K., Irie, K., Klein, W. L., Mori, H., Asada, T., Takahashi, R., Iwata, N., Yamanaka, S. and Inoue, H. (2013) 'Modeling Alzheimer's Disease with iPSCs Reveals Stress Phenotypes Associated with Intracellular Aβ and Differential Drug Responsiveness', *Cell Stem Cell*, 12(4), pp. 487–496. doi: 10.1016/j.stem.2013.01.009.

Kraus, D., Boyle, V., Leibig, N., Stark, G. and Penna, V. (2015) 'The Neuro-spheroid—A novel 3D in vitro model for peripheral nerve regeneration', *Journal of Neuroscience Methods*, 246, pp. 97–105. doi: 10.1016/j.jneumeth.2015.03.004.

Krencik, R. and Ullian, E. M. (2013) 'A cellular star atlas: using astrocytes from human pluripotent stem cells for disease studies', *Frontiers in Cellular Neuroscience*, 7(March), p. 25.

doi: 10.3389/fncel.2013.00025.

Lagutina, I., Fulka, H., Lazzari, G. and Galli, C. (2013) ‘Interspecies somatic cell nuclear transfer: advancements and problems.’, *Cellular reprogramming*, 15(5), pp. 374–84. doi: 10.1089/cell.2013.0036.

Lappalainen, R. S., Salomäki, M., Ylä-Outinen, L., Heikkilä, T. J., Hyttinen, J. a K., Pihlajamäki, H., Suuronen, R., Skottman, H. and Narkilahti, S. (2010) ‘Similarly derived and cultured hESC lines show variation in their developmental potential towards neuronal cells in long-term culture’, *Regenerative medicine*, 5(5), pp. 749–762. doi: 10.2217/rme.10.58.

Lau, H.-T., Liu, L., Ray, C., Bell, F. T. and Li, X. (2016) ‘Derivation of hybrid ES cell lines from two different strains of mice’, *Stem Cell Research*, 16(2), pp. 252–255. doi: 10.1016/j.scr.2016.01.017.

Lee, G., Papapetrou, E. P., Kim, H., Chambers, S. M., Tomishima, M. J., Fasano, C. A., Ganat, Y. M., Menon, J., Shimizu, F., Viale, A., Tabar, V., Sadelain, M. and Studer, L. (2009) ‘Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs’, *Nature*, 461(7262), pp. 402–406. doi: 10.1038/nature08320.

Lee, O. K., Kuo, T. K., Chen, W.-M., Lee, K.-D., Hsieh, S.-L. and Chen, T.-H. (2004) ‘Isolation of multipotent mesenchymal stem cells from umbilical cord blood.’, *Blood*, 103(5), pp. 1669–75. doi: 10.1182/blood-2003-05-1670.

Leehee, W., Ayyash, M., Novershtern, N. and H., H. J. (2016) ‘Dynamic stem cell states: naive to primed pluripotency in rodents and humans’, *Nature Reviews Molecular Cell Biology*, 17(3), pp. 155–169. doi: 10.1038/nrm.2015.28.

Li, T., Lewallen, M., Chen, S., Yu, W., Zhang, N. and Xie, T. (2013) ‘Multipotent stem cells isolated from the adult mouse retina are capable of producing functional photoreceptor cells.’, *Cell research*, 23(6), pp. 788–802. doi: 10.1038/cr.2013.48.

Li, W. and Ding, S. (2011) ‘Human pluripotent stem cells: decoding the naïve state.’, *Science translational medicine*, 3(76), p. 76ps10. doi: 10.1126/scitranslmed.3000996.

Li, W., Wei, W., Zhu, S., Zhu, J., Shi, Y., Lin, T., Hao, E., Hayek, A., Deng, H. and Ding, S. (2009) ‘Generation of Rat and Human Induced Pluripotent Stem Cells by Combining Genetic Reprogramming and Chemical Inhibitors (DOI:10.1016/j.stem.2008.11.014)’, *Cell Stem Cell*, p. 370. doi: 10.1016/j.stem.2009.03.002.

Liem, K. F., Tremml, G., Roelink, H. and Jessell, T. M. (1995) ‘Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm’, *Cell*, 82(6), pp. 969–979. doi: 10.1016/0092-8674(95)90276-7.

Lin, S.-L., Chang, D. C., Chang-Lin, S., Lin, C.-H., Wu, D. T. S., Chen, D. T. and Ying, S.-Y. (2008) ‘Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state.’, *RNA (New York, N.Y.)*, 14(10), pp. 2115–24. doi: 10.1261/rna.1162708.

Liu, H. and Zhang, S.-C. (2011) ‘Specification of neuronal and glial subtypes from human pluripotent stem cells.’, *Cellular and molecular life sciences : CMLS*, 68(24), pp. 3995–4008. doi: 10.1007/s00018-011-0770-y.

Liu, M.-L., Zang, T. and Zhang, C.-L. (2016) ‘Direct Lineage Reprogramming Reveals Disease-Specific Phenotypes of Motor Neurons from Human ALS Patients’, *Cell Reports*, 14(1), pp. 115–128. doi: 10.1016/j.celrep.2015.12.018.

Liu, Y., Liu, H., Sauvey, C., Yao, L., Zarnowska, E. D. and Zhang, S.-C. (2013) ‘Directed differentiation of forebrain GABA interneurons from human pluripotent stem cells.’, *Nature protocols*, 8(9), pp. 1670–9. doi: 10.1038/nprot.2013.106.

Louvi, A. and Artavanis-Tsakonas, S. (2006) 'Notch signalling in vertebrate neural development.', *Nature reviews. Neuroscience*, 7(2), pp. 93–102. doi: 10.1038/nrn1847.

Lundkvist, J. and Lendahl, U. (2001) 'Notch and the birth of glial cells', *Trends in Neurosciences*, pp. 492–494. doi: 10.1016/S0166-2236(00)01888-9.

Macvicar, B. A. and Newman, E. A. (2015) 'Astrocyte Regulation of Blood Flow in the Brain', *Cold Spring Harbor perspectives in biology*, 7, pp. 1–14. doi: 10.1101/cshperspect.a020388.

Magistretti, P. J., Pellerin, L., Rothman, D. L. and Shulman, R. G. (1999) 'Energy on Demand', *Science*, 283(5401), pp. 496–497. doi: 10.1126/science.283.5401.496.

Mandai, M., Watanabe, A., Kurimoto, Y., Hirami, Y., Morinaga, C., Daimon, T., Fujihara, M., Akimaru, H., Sakai, N., Shibata, Y., Terada, M., Nomiya, Y., Tanishima, S., Nakamura, M., Kamao, H., Sugita, S., Onishi, A., Ito, T., Fujita, K., Kawamata, S., Go, M. J., Shinohara, C., Hata, K., Sawada, M., Yamamoto, M., Ohta, S., Ohara, Y., Yoshida, K., Kuwahara, J., Kitano, Y., Amano, N., Umekage, M., Kitaoka, F., Tanaka, A., Okada, C., Takasu, N., Ogawa, S., Yamanaka, S. and Takahashi, M. (2017) 'Autologous Induced Stem-Cell–Derived Retinal Cells for Macular Degeneration', *New England Journal of Medicine*. Massachusetts Medical Society, 376(11), pp. 1038–1046. doi: 10.1056/NEJMoa1608368.

Marchetto, M. C. N., Carromeu, C., Acab, A., Yu, D., Yeo, G. W., Mu, Y., Chen, G., Gage, F. H. and Muotri, A. R. (2010) 'A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells.', *Cell*. NIH Public Access, 143(4), pp. 527–39. doi: 10.1016/j.cell.2010.10.016.

Mariani, J., Vittoria, M., Palejev, D. and Tomasini, Livia; Coppola, G.; Szekely, A.M.; Horvath, T.L.; Vaccarino, M. V. (2012) 'Modeling human cortical development in vitro using induced pluripotent stem cells', *Proceedings of the National Academy of Sciences*, 109(31), pp. 12770–12775. doi: 10.1073/pnas.1202944109/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1202944109.

Martin, G. R. (1981) 'Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.', *Proceedings of the National Academy of Sciences of the United States of America*, 78(12), pp. 7634–7638. doi: 10.1073/pnas.78.12.7634.

Massagué, J. (2012) 'TGFβ signalling in context.', *Nature reviews. Molecular cell biology*, 13(10), pp. 616–30. doi: 10.1038/nrm3434.

McEwen, K. R., Leitch, H. G., Amouroux, R. and Hajkova, P. (2013) 'The impact of culture on epigenetic properties of pluripotent stem cells and pre-implantation embryos', *Biochem Soc Trans*, 41(3), pp. 711–719. doi: 10.1042/bst20130049.

Mestas, J. and Hughes, C. C. W. (2004) 'Of mice and not men: differences between mouse and human immunology.', *Journal of immunology (Baltimore, Md. : 1950)*, 172(5), pp. 2731–2738. doi: 10.4049/jimmunol.172.5.2731.

Meyer-Franke, Kaplan, M. R., Pfrieger, F. W. and Barres, B. (1995) 'Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture.', *Neuron*, 15(4), pp. 805–819. doi: 10.1016/0896-6273(95)90172-8.

Mitchell, L. E., Scott Adzick, N., Melchionne, J., Pasquariello, P. S., Sutton, L. N. and Whitehead, A. S. (2004) 'Spina bifida', in *Lancet*, pp. 1885–1895. doi: 10.1016/S0140-6736(04)17445-X.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M. and Yamanaka, S. (2003) 'The homeoprotein nanog is required for maintenance of pluripotency in mouse epiblast and ES cells', *Cell*, 113(5), pp. 631–642. doi: 10.1016/S0092-8674(03)00393-3.

Molyneaux, B. J., Arlotta, P., Menezes, J. R. L. and Macklis, J. D. (2007) 'Neuronal subtype specification in the cerebral cortex.', *Nature Reviews. Neuroscience*, 8(6), pp. 427–37. doi: 10.1038/nrn2151.

Momcilovic, O., Sivapatham, R., Oron, T. R., Meyer, M., Mooney, S., Rao, M. S. and Zeng, X. (2016) 'Derivation, Characterization, and Neural Differentiation of Integration-Free Induced Pluripotent Stem Cell Lines from Parkinson's Disease Patients Carrying SNCA, LRRK2, PARK2, and GBA Mutations', *PloS one*, 11(5), p. e0154890. doi: 10.1371/journal.pone.0154890.

Moon, C., Yoo, J.-Y., Matarazzo, V., Sung, Y. K., Kim, E. J. and Ronnett, G. V (2002) 'Leukemia inhibitory factor inhibits neuronal terminal differentiation through STAT3 activation', *Proceedings of the National Academy of Sciences of the United States of America*, 99(13), pp. 9015–20. doi: 10.1073/pnas.132131699.

Muguruma, K. and Sasai, Y. (2012) 'In vitro recapitulation of neural development using embryonic stem cells: From neurogenesis to histogenesis', *Development Growth and Differentiation*, 54(3), pp. 349–357. doi: 10.1111/j.1440-169X.2012.01329.x.

Muñoz-Sanjuán, I. and Brivanlou, A. (2002) 'Neural induction, the default model and embryonic stem cells', *Nature Reviews Neuroscience*, 3(4), pp. 271–280. doi: 10.1038/nrn786.

Munsie, M. J., Michalska, A. E., O'Brien, C. M., Trounson, A. O., Pera, M. F. and Mountford, P. S. (2000) 'Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei', *Current Biology*, pp. 989–992. doi: 10.1016/S0960-9822(00)00648-5.

Muratore, C. R., Rice, H. C., Srikanth, P., Callahan, D. G., Shin, T., Benjamin, L. N. P., Walsh, D. M., Selkoe, D. J. and Young-Pearse, T. L. (2014) 'The familial alzheimer's disease APPV717I mutation alters APP processing and Tau expression in iPSC-derived neurons', *Human Molecular Genetics*, 23(13), pp. 3523–3536. doi: 10.1093/hmg/ddu064.

Muratore, C. R., Srikanth, P., Callahan, D. G. and Young-Pearse, T. L. (2014) 'Comparison and optimization of hiPSC forebrain cortical differentiation protocols', *PLoS ONE*, 9(8). doi: 10.1371/journal.pone.0105807.

Musina, R. A., Bekchanova, E. S., Belyavskii, A. V., Grinenko, T. S. and Sukhikh, G. T. (2007) 'Umbilical cord blood mesenchymal stem cells', *Bulletin of Experimental Biology and Medicine*, 143(1), pp. 127–131. doi: 10.1007/s10517-007-0032-z.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. and Roder, J. C. (1993) 'Derivation of completely cell culture-derived mice from early-passage embryonic stem cells.', *Proceedings of the National Academy of Sciences of the United States of America*, 90(18), pp. 8424–8. doi: 10.1073/pnas.90.18.8424.

Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N. and Yamanaka, S. (2008) 'Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts.', *Nature biotechnology*, 26(1), pp. 101–6. doi: 10.1038/nbt1374.

Nedergaard, M., Ransom, B. and Goldman, S. A. (2003) 'New roles for astrocytes: Redefining the functional architecture of the brain', *Trends in Neurosciences*, pp. 523–530. doi: 10.1016/j.tins.2003.08.008.

Nemes, C., Varga, E., Polgar, Z., Klincumhom, N., Pirity, M. K. and Dinnyes, A. (2014) 'Generation of mouse induced pluripotent stem cells by protein transduction.', *Tissue engineering. Part C, Methods*. Mary Ann Liebert, Inc., 20(5), pp. 383–92. doi: 10.1089/ten.TEC.2013.0026.

Nemes, C., Varga, E., Táncos, Z., Bock, I., Francz, B., Kobolák, J. and Dinnyés, A. (2016)

'Establishment of PSEN1 mutant induced pluripotent stem cell (iPSC) line from an Alzheimer's disease (AD) female patient', *Stem Cell Research*, 17(1), pp. 69–71. doi: 10.1016/j.scr.2016.05.019.

Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S. and Koopman, P. (1997) 'SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse.', *Developmental biology*, 183, pp. 108–121. doi: 10.1006/dbio.1996.8487.

Nguyen, H. N., Byers, B., Cord, B., Shcheglovitov, A., Byrne, J., Gujar, P., Kee, K., Schüle, B., Dolmetsch, R. E., Langston, W., Palmer, T. D. and Pera, R. R. (2011) 'LRRK2 mutant iPSC-derived da neurons demonstrate increased susceptibility to oxidative stress', *Cell Stem Cell*, 8(3), pp. 267–280. doi: 10.1016/j.stem.2011.01.013.

Nichols, J. and Smith, A. (2009) 'Naive and Primed Pluripotent States', *Cell Stem Cell*, pp. 487–492. doi: 10.1016/j.stem.2009.05.015.

Niu, W., Zang, T., Smith, D. K., Vue, T. Y., Zou, Y., Bachoo, R., Johnson, J. E. and Zhang, C. L. (2015) 'SOX2 reprograms resident astrocytes into neural progenitors in the adult brain', *Stem Cell Reports*, 4(5), pp. 780–794. doi: 10.1016/j.stemcr.2015.03.006.

Niwa, H., Toyooka, Y., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, R. and Rossant, J. (2005) 'Interaction between Oct3/4 and Cdx2 Determines Trophectoderm Differentiation', *Cell*, 123(5), pp. 917–929. doi: 10.1016/j.cell.2005.08.040.

O'Connor, S. M., Stenger, D. A., Shaffer, K. M., Maric, D., Barker, J. L. and Ma, W. (2000) 'Primary neural precursor cell expansion, differentiation and cytosolic Ca(2+) response in three-dimensional collagen gel.', *Journal of neuroscience methods*, 102(2), pp. 187–195. doi: 10.1016/S0165-0270(00)00303-4.

O'Donoghue, K. and Fisk, N. M. (2004) 'Fetal stem cells', *Best Practice and Research: Clinical Obstetrics and Gynaecology*, 18(6), pp. 853–875. doi: 10.1016/j.bpobgyn.2004.06.010.

O'Rahilly, R. and Muller, F. (1994) 'Neurulation in the normal human embryo', *Ciba Found Symp*, 181, pp. 70–79. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8005032.

Oberheim, N. A., Wang, X., Goldman, S. and Nedergaard, M. (2006) 'Astrocytic complexity distinguishes the human brain', *Trends in Neurosciences*, 29(10), pp. 547–553. doi: 10.1016/j.tins.2006.08.004.

Ochalek, A., Nemes, C., Varga, E., Táncos, Z., Kobolák, J. and Dinnyés, A. (2016) 'Establishment of induced pluripotent stem cell (iPSC) line from a 57-year old patient with sporadic Alzheimer's disease', *Stem Cell Research*, 17(1), pp. 72–74. doi: 10.1016/j.scr.2016.05.020.

Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Hong, H., Nakagawa, M., Tanabe, K., Tezuka, K., Shibata, T., Kunisada, T., Takahashi, M., Takahashi, J., Saji, H. and Yamanaka, S. (2011) 'A more efficient method to generate integration-free human iPS cells', *Nature methods*, 8(5), pp. 409–412. doi: 10.1038/nmeth.1591.

Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T. and Shinya Yamanaka (2008) 'Generation of Mouse Induced Pluripotent Stem Cells Without Viral Vectors', *Science*, 322(1998), pp. 949–953. doi: 10.1126/science.1164270.

Onorati, M., Castiglioni, V., Biasci, D., Cesana, E., Menon, R., Vuono, R., Talpo, F., Goya, R. L., Lyons, P. A., Bulfamante, G. P., Muzio, L., Martino, G., Toselli, M., Farina, C., Barker, R. A., Biella, G. and Cattaneo, E. (2014) 'Molecular and functional definition of the developing human striatum', *Nat Neurosci*, 17(12), pp. 1804–1815. doi: 10.1038/nn.3860.

Oshimori, N. and Fuchs, E. (2012) ‘Paracrine TGF-β signaling counterbalances BMP-mediated repression in hair follicle stem cell activation’, *Cell Stem Cell*, 10(1), pp. 63–75. doi: 10.1016/j.stem.2011.11.005.

Osumi, N. (2001) ‘The role of Pax6 in brain patterning’, *Tohoku J Exp Med*, 193(3), pp. 163–174. doi: 10.1620/tjem.193.163.

Oswald, J., Boxberger, S., Jorgensen, B., Feldmann, S., Ehninger, G., Bornhauser, M. and Werner, C. (2004) ‘Mesenchymal stem cells can be differentiated into endothelial cells in vitro’, *Stem Cells*, 22, pp. 377–384. doi: DOI 10.1634/stemcells.22-3-377.

Pandey, U. B. and Nichols, C. D. (2011) ‘Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery.’, *Pharmacological reviews*. American Society for Pharmacology and Experimental Therapeutics, 63(2), pp. 411–36. doi: 10.1124/pr.110.003293.

Pankratz, M. T., Li, X.-J., LaVaute, T. M., Lyons, E. A., Chen, X. and Zhang, S.-C. (2007) ‘Directed Neural Differentiation of Human Embryonic Stem Cells via an Obligated Primitive Anterior Stage’, *Stem Cells*, 25(6), pp. 1511–1520. doi: 10.1634/stemcells.2006-0707.

Papapetrou, E. P., Tomishima, M. J., Chambers, S. M., Mica, Y., Reed, E., Menon, J., Tabar, V., Mo, Q., Studer, L. and Sadelain, M. (2009) ‘Stoichiometric and temporal requirements of Oct4, Sox2, Klf4, and c-Myc expression for efficient human iPSC induction and differentiation.’, *Proceedings of the National Academy of Sciences of the United States of America*, 106(31), pp. 12759–64. doi: 10.1073/pnas.0904825106.

Parameswaran, S., Balasubramanian, S., Babai, N., Qiu, F., Eudy, J. D., Thoreson, W. B. and Ahmad, I. (2010) ‘Induced pluripotent stem cells generate both retinal ganglion cells and photoreceptors: Therapeutic implications in degenerative changes in glaucoma and age-related macular degeneration’, *Stem Cells*, 28(4), pp. 695–703. doi: 10.1002/stem.320.

Parpura, V. and Verkhratsky, A. (2012) ‘Astrocytes revisited: concise historic outlook on glutamate homeostasis and signaling.’, *Croatian medical journal*, 53(6), pp. 518–28. doi: 10.3325/cmj.2012.53.518.

Paşca, A. M., Sloan, S. A., Clarke, L. E., Tian, Y., Makinson, C. D., Huber, N., Kim, C. H., Park, J.-Y., O’Rourke, N. A., Nguyen, K. D., Smith, S. J., Huguenard, J. R., Geschwind, D. H., Barres, B. A. and Paşca, S. P. (2015) ‘Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture’, *Nature Methods*, 12(7), pp. 671–8. doi: 10.1038/nmeth.3415.

Patsch, C., Challet-Meylan, L., Thoma, E. C., Urich, E., Heckel, T., O’Sullivan, J. F., Grainger, S. J., Kapp, F. G., Sun, L., Christensen, K., Xia, Y., Florido, M. H. C., He, W., Pan, W., Prummer, M., Warren, C. R., Jakob-Roetne, R., Certa, U., Jagasia, R., Freskgård, P.-O., Adatto, I., Kling, D., Huang, P., Zon, L. I., Chaikof, E. L., Gerszten, R. E., Graf, M., Iacone, R. and Cowan, C. A. (2015) ‘Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells’, *Nature Cell Biology*. Nature Research, 17(8), pp. 994–1003. doi: 10.1038/ncb3205.

Paulson, O. B. and Newman, E. A. (1987) ‘Does the release of potassium from astrocyte endfeet regulate cerebral blood flow?’, *Science (New York, N.Y.)*, 237(4817), pp. 896–8. doi: 10.1016/j.bbi.2008.05.010.

Pellerin, L., Pellegri, G., Bittar, P. G., Charnay, Y., Bouras, C., Martin, J. L., Stella, N. and Magistretti, P. J. (1998) ‘Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle’, *Developmental Neuroscience*, 20(4–5), pp. 291–299. doi: 10.1159/000017324.

Pen, A. E. and Jensen, U. B. (2016) ‘Current status of treating neurodegenerative disease with induced pluripotent stem cells’, *Acta Neurologica Scandinavica*, (1), pp. 1–16. doi: 10.1111/ane.12545.

Pera, M. F., Reubinoff, B. and Trounson, A. (2000) ‘Human embryonic stem cells’, *Journal of Cell Science*, 113, pp. 5–10. doi: 10.1002/(sici)1097-0215(19960611)66:6<806::aid-ijc17>3.0.co.

Perrier, A. L., Tabar, V., Barberi, T., Rubio, M. E., Bruses, J., Topf, N., Harrison, N. L. and Studer, L. (2004) ‘Derivation of midbrain dopamine neurons from human embryonic stem cells.’, *Proceedings of the National Academy of Sciences of the United States of America*, 101(34), pp. 12543–12548. doi: 10.1073/pnas.0404700101.

Petros, T. J., Tyson, J. A. and Anderson, S. A. (2011) ‘Pluripotent Stem Cells for the Study of CNS Development’, *Frontiers in Molecular Neuroscience*. Frontiers, 4, p. 30. doi: 10.3389/fnmol.2011.00030.

Pool, M., Thiemann, J., Bar-Or, A. and Fournier, A. E. (2008) ‘NeuriteTracer: A novel ImageJ plugin for automated quantification of neurite outgrowth’, *Journal of Neuroscience Methods*, 168(1), pp. 134–139. doi: 10.1016/j.jneumeth.2007.08.029.

Pouya, A., Satarian, L., Kiani, S., Javan, M. and Baharvand, H. (2011) ‘Human induced pluripotent stem cells differentiation into oligodendrocyte progenitors and transplantation in a rat model of optic chiasm demyelination.’, *PloS one*, 6(11), p. e27925. doi: 10.1371/journal.pone.0027925.

Prust, M., Wang, J., Morizono, H., Messing, a, Brenner, M., Gordon, E., Hartka, T., Sokohl, a, Schiffmann, R., Gordish-Dressman, H., Albin, R., Amartino, H., Brockman, K., Dinopoulos, a, Dotti, M. T., Fain, D., Fernandez, R., Ferreira, J., Fleming, J., Gill, D., Griebel, M., Heilstedt, H., Kaplan, P., Lewis, D., Nakagawa, M., Pedersen, R., Reddy, a, Sawaishi, Y., Schneider, M., Sherr, E., Takiyama, Y., Wakabayashi, K., Gorospe, J. R. and Vanderver, a (2011) ‘GFAP mutations, age at onset, and clinical subtypes in Alexander disease.’, *Neurology*, 77(13), pp. 1287–94. doi: 10.1212/WNL.0b013e3182309f72.

Rajala, K., Hakala, H., Panula, S., Aivio, S., Pihlajamäki, H., Suuronen, R., Hovatta, O. and Skottman, H. (2007) ‘Testing of nine different xeno-free culture media for human embryonic stem cell cultures’, *Human Reproduction*, 22(5), pp. 1231–1238. doi: 10.1093/humrep/del523.

Ranganatha, N. and Kuppast, I. J. (2012) ‘A review on alternatives to animal testing methods in drug development’, *International Journal of Pharmacy and Pharmaceutical Sciences*, 4(SUPPL. 5), pp. 28–32.

Reubinoff, B. E., Itsykson, P., Turetsky, T., Pera, M. F., Reinhartz, E., Itzik, A. and Ben-Hur, T. (2001) ‘Neural progenitors from human embryonic stem cells’, *Nat Biotechnol*, 19(12), p. 1134–40. doi: 10.1038/nbt1201-1134.

Rhee, Y. H., Ko, J. Y., Chang, M. Y., Yi, S. H., Kim, D., Kim, C. H., Shim, J. W., Jo, A. Y., Kim, B. W., Lee, H., Lee, S. H., Suh, W., Park, C. H., Koh, H. C., Lee, Y. S., Lanza, R., Kim, K. S. and Lee, S. H. (2011) ‘Protein-based human iPS cells efficiently generate functional dopamine neurons and can treat a rat model of Parkinson disease’, *Journal of Clinical Investigation*, 121(6), pp. 2326–2335. doi: 10.1172/JCI45794.

Ribeiro, A., Vargo, S., Powell, E. M. and Leach, J. B. (2012) ‘Substrate Three-Dimensionality Induces Elemental Morphological Transformation of Sensory Neurons on a Physiologic Timescale’, *Tissue Engineering Part A*, 18(1–2), pp. 93–102. doi: 10.1089/ten.tea.2011.0221.

Ricci, G., Volpi, L., Pasquali, L., Petrozzi, L. and Siciliano, G. (2009) ‘Astrocyte-neuron interactions in neurological disorders’, *Journal of Biological Physics*, 35, pp. 317–336. doi:

10.1007/s10867-009-9157-9.

Robinton, D. A. and Daley, G. Q. (2012) ‘The promise of induced pluripotent stem cells in research and therapy’, *Nature*, 481(7381), pp. 295–305. doi: 10.1038/nature10761.

Rogers, I. and Casper, R. F. (2004) ‘Umbilical cord blood stem cells.’, *Best practice & research. Clinical obstetrics & gynaecology*, 18(6), pp. 893–908. doi: 10.1016/j.bpobgyn.2004.06.004.

Rooney, G. E., Goodwin, A. F., Depeille, P., Sharir, A., Schofield, C. M., Yeh, E., Roose, J. P., Klein, O. D., Rauen, K. A., Weiss, L. A. and Ullian, E. M. (2016) ‘Human iPS Cell-Derived Neurons Uncover the Impact of Increased Ras Signaling in Costello Syndrome.’, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 36(1), pp. 142–52. doi: 10.1523/JNEUROSCI.1547-15.2016.

Rowland, B. D. and Peeper, D. S. (2006) ‘KLF4, p21 and context-dependent opposing forces in cancer.’, *Nature reviews. Cancer*, 6(1), pp. 11–23. doi: 10.1038/nrc1780.

Rowland, L. and Shneider, N. (2001) ‘Amyotrophic lateral sclerosis’, *New England Journal of Medicine*, 344(22), pp. 1688–1700. doi: 10.1056/NEJM200105313442207.

Sakai, Y. (1989) ‘Neurulation in the mouse: Manner and timing of neural tube closure’, *The Anatomical Record*, 223(2), pp. 194–203. doi: 10.1002/ar.1092230212.

Salguero-Aranda, C., Tapia-Limonchi, R., Cahuana, G. M., Hitos, A. B., Diaz, I., Hmadcha, A., Fraga, M., Martin, F., Soria, B., Tejedo, J. R. and Bedoya, F. J. (2016) ‘Differentiation of Mouse Embryonic Stem Cells Towards Functional Pancreatic Beta-Cell Surrogates through Epigenetic Regulation of Pdx1 by Nitric Oxide’, *Cell Transplant*. doi: 10.3727/096368916X691178.

Sánchez-Danés, A., Consiglio, A., Richaud, Y., Rodríguez-Pizà, I., Dehay, B., Edel, M., Bové, J., Memo, M., Vila, M., Raya, A. and Izpisua Belmonte, J. C. (2012) ‘Efficient Generation of A9 Midbrain Dopaminergic Neurons by Lentiviral Delivery of LMX1A in Human Embryonic Stem Cells and Induced Pluripotent Stem Cells’, *Human Gene Therapy*, 23(1), pp. 56–69. doi: 10.1089/hum.2011.054.

Sant’Anna, J. R. de, Franco, C. C. da S., Mathias, P. C. de F., Castro-Prado, M. A. A. de, Phonethepswath, S. and Labash, C. (2015) ‘Assessment of In Vivo and In Vitro Genotoxicity of Glibenclamide in Eukaryotic Cells’, *PLOS ONE*. Edited by A. Ahmad. Public Library of Science, 10(3), p. e0120675. doi: 10.1371/journal.pone.0120675.

Santoriello, C. and Zon, L. I. (2012) ‘Hooked! Modeling human disease in zebrafish’, *Journal of Clinical Investigation*, 122(7), pp. 2337–2343. doi: 10.1172/JCI60434.

Saporta, M. A., Grskovic, M. and Dimos, J. T. (2011) ‘Induced pluripotent stem cells in the study of neurological diseases’, *Stem Cell Research & Therapy*, 2(5), p. 37. doi: 10.1186/scrt78.

Sartori, C., DiDomenico, A. I., Thomson, A. J., Milne, E., Lillico, S. G., Burdon, T. G. and Whitelaw, C. B. a (2012) ‘Ovine-induced pluripotent stem cells can contribute to chimeric lambs.’, *Cellular reprogramming*, 14(1), pp. 8–19. doi: 10.1089/cell.2011.0050.

Schnerch, A., Cerdan, C. and Bhatia, M. (2010) ‘Distinguishing between mouse and human pluripotent stem cell regulation: The best laid plans of mice and men’, *Stem Cells*, pp. 419–430. doi: 10.1002/stem.298.

Schulz, T. C., Young, H. Y., Agulnick, A. D., Babin, M. J., Baetge, E. E., Bang, A. G., Bhoumik, A., Cepa, I., Cesario, R. M., Haakmeester, C., Kadoya, K., Kelly, J. R., Kerr, J., Martinson, L. A., McLean, A. B., Moorman, M. A., Payne, J. K., Richardson, M., Ross, K. G., Sherrer, E. S., Song, X., Wilson, A. Z., Brandon, E. P., Green, C. E., Kroon, E. J., Kelly, O. G., D’Amour, K. A. and Robins, A. J. (2012) ‘A scalable system for production of functional pancreatic progenitors from human embryonic stem cells’, *PLoS ONE*, 7(5). doi:

10.1371/journal.pone.0037004.

Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W. and Selbach, M. (2011) 'Global quantification of mammalian gene expression control', *Nature*, 473(7347), pp. 337–342. doi: nature10098 [pii]10.1038/nature10098.

Schwartz, S. D., Regillo, C. D., Lam, B. L., Eliott, D., Rosenfeld, P. J., Gregori, N. Z., Hubschman, J.-P., Davis, J. L., Heilwell, G., Spirn, M., Maguire, J., Gay, R., Bateman, J., Ostrick, R. M., Morris, D., Vincent, M., Anglade, E., Del Priore, L. V and Lanza, R. (2015) 'Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies', *The Lancet*, 385(9967), pp. 509–516. doi: 10.1016/S0140-6736(14)61376-3.

Scott, C. E., Wynn, S. L., Sesay, A., Cruz, C., Cheung, M., Gaviro, M.-V. G., Booth, S., Gao, B., Cheah, K. S. E., Lovell-Badge, R. and Briscoe, J. (2010) 'SOX9 induces and maintains neural stem cells', *Nature Neuroscience*. Nature Research, 13(10), pp. 1181–1189. doi: 10.1038/nn.2646.

Scott C, Wynn S, Sesay A, Cruz C, Cheung M, Gaviro M, Booth, Gao B, Cheah K, L.-B. R. B. J. (2010) 'SOX9 induces and maintains neural stem cells', *nature NEUROSCIENCE*, 13(10), pp. 1181–90. doi: 10.1038/nn.2646.

Seibler, P., Graziotto, J., Jeong, H., Simunovic, F., Klein, C. and Krainc, D. (2011) 'Mitochondrial Parkin recruitment is impaired in neurons derived from mutant PINK1 induced pluripotent stem cells.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 31(16), pp. 5970–6. doi: 10.1523/JNEUROSCI.4441-10.2011.

Shi, Y., Kirwan, P. and Livesey, F. J. (2012) 'Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks', *Nature Protocols*, 7(10), pp. 1836–1846. doi: 10.1038/nprot.2012.116.

Shi, Y., Kirwan, P., Smith, J., Robinson, H. P. and Livesey, F. J. (2012) 'Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses', *Nat Neurosci*, 15(3), pp. 477–86, S1. doi: nn.3041 [pii]\n10.1038/nn.3041.

Shimojo, D., Onodera, K., Doi-Torii, Y., Ishihara, Y., Hattori, C., Miwa, Y., Tanaka, S., Okada, R., Ohyama, M., Shoji, M., Nakanishi, A., Doyu, M., Okano, H. and Okada, Y. (2015) 'Rapid, efficient, and simple motor neuron differentiation from human pluripotent stem cells.', *Molecular brain*, 8(1), p. 79. doi: 10.1186/s13041-015-0172-4.

Shroff, G. and Gupta, R. (2015) 'Human embryonic stem cells in the treatment of patients with spinal cord injury.', *Annals of neurosciences*. Karger Publishers, 22(4), pp. 208–16. doi: 10.5214/ans.0972.7531.220404.

Sidoryk-Wegrzynowicz, M., Wegrzynowicz, M., Lee, E., Bowman, A. B. and Aschner, M. (2011) 'Role of astrocytes in brain function and disease.', *Toxicologic pathology*, 39(1), pp. 115–123. doi: 10.1177/0192623310385254.

Siller, R., Greenhough, S., Park, I.-H. and Sullivan, G. J. (2013) 'Modelling human disease with pluripotent stem cells.', *Current gene therapy*. NIH Public Access, 13(2), pp. 99–110. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23444871 (Accessed: 4 November 2016).

Slezak, M. and Pfrieger, F. W. (2003) 'New roles for astrocytes: Regulation of CNS synaptogenesis', *Trends in Neurosciences*, pp. 531–535. doi: 10.1016/j.tins.2003.08.005.

Smith, a G. (2001) 'Embryo-derived stem cells: of mice and men.', *Annual review of cell and developmental biology*, 17, pp. 435–62. doi: 10.1146/annurev.cellbio.17.1.435.

Sollazzo, V., Palmieri, A., Girardi, A., Farinella, F. and Carinci, F. (2011) 'Biotechnology &

Biomaterials Trabecular Titanium Induces Osteoblastic Bone Marrow Stem Cells Differentiation', *Journal of Biotechnology*, 1(1), pp. 1–5. doi: 10.4172/2155-952X.1000102.

Song, W. K., Park, K.-M., Kim, H.-J., Lee, J. H., Choi, J., Chong, S. Y., Shim, S. H., Del Priore, L. V and Lanza, R. (2015) 'Treatment of macular degeneration using embryonic stem cell-derived retinal pigment epithelium: preliminary results in Asian patients.', *Stem cell reports*. Elsevier, 4(5), pp. 860–72. doi: 10.1016/j.stemcr.2015.04.005.

Sproul, A. A., Jacob, S., Pre, D., Kim, S. H., Nestor, M. W., Navarro-Sobrino, M., Santa-Maria, I., Zimmer, M., Aubry, S., Steele, J. W., Kahler, D. J., Dranovsky, A., Arancio, O., Crary, J. F., Gandy, S. and Noggle, S. A. (2014) 'Characterization and molecular profiling of PSEN1 familial alzheimer's disease iPSC-Derived neural progenitors', *PLoS ONE*, 9(1). doi: 10.1371/journal.pone.0084547.

Stevens, B., Allen, N. J., Vazquez, L. E., Howell, G. R., Christopherson, K. S., Nouri, N., Micheva, K. D., Mehalow, A. K., Huberman, A. D., Stafford, B., Sher, A., Litke, A. M., Lambris, J. D., Smith, S. J., John, S. W. M. and Barres, B. A. (2007) 'The Classical Complement Cascade Mediates CNS Synapse Elimination', *Cell*, 131(6), pp. 1164–1178. doi: 10.1016/j.cell.2007.10.036.

Stiles, J. and Jernigan, T. (2010) 'The Basics of Brain Development', *Neuropsychology Review*, 20(4), pp. 327–348. doi: 10.1007/s11065-010-9148-4.

Stipursky, J., Francis, D. and Gomes, F. C. A. (2012) 'Activation of MAPK/PI3K/SMAD pathways by TGF-β1 controls differentiation of radial glia into astrocytes in vitro', *Developmental Neuroscience*, 34(1), pp. 68–81. doi: 10.1159/000338108.

Stipursky, J., Romão, L., Tortelli, V., Neto, V. M. and Gomes, F. C. A. (2011) 'Neuron-glia signaling: Implications for astrocyte differentiation and synapse formation', in *Life Sciences*, pp. 524–531. doi: 10.1016/j.lfs.2011.04.005.

Stobart, J. L. and Anderson, C. M. (2013) 'Multifunctional role of astrocytes as gatekeepers of neuronal energy supply.', *Frontiers in cellular neuroscience*, 7(April), p. 38. doi: 10.3389/fncel.2013.00038.

Stolt, C. C., Lommes, P., Sock, E., Chaboissier, M. C., Schedl, A. and Wegner, M. (2003) 'The Sox9 transcription factor determines glial fate choice in the developing spinal cord', *Genes and Development*, 17(13), pp. 1677–1689. doi: 10.1101/gad.259003.

Strowig, T., Chijioke, O., Carrega, P., Arrey, F., Meixlsperger, S., Rämer, P. C., Ferlazzo, G. and Münz, C. (2010) 'Human NK cells of mice with reconstituted human immune system components require preactivation to acquire functional competence.', *Blood*. American Society of Hematology, 116(20), pp. 4158–67. doi: 10.1182/blood-2010-02-270678.

Strzyz, P. (2016) 'Adult stem cells: Hair stem cells born without a home', *Nature Reviews Molecular Cell Biology*. Nature Research, 17(3), pp. 133–133. doi: 10.1038/nrm.2016.9.

Sun, G., Liu, W., Fan, Z., Zhang, D., Han, Y., Xu, L., Qi, J., Zhang, S., Gao, B. T., Bai, X., Li, J., Chai, R. and Wang, H. (2016) 'The Three-Dimensional Culture System with Matrigel and Neurotrophic Factors Preserves the Structure and Function of Spiral Ganglion Neuron in Vitro', *Neural Plasticity*, 2016. doi: 10.1155/2016/4280407.

Sundberg, M., Jansson, L., Ketolainen, J., Pihlajamäki, H., Suuronen, R., Skottman, H., Inzunza, J., Hovatta, O. and Narkilahti, S. (2009) 'CD marker expression profiles of human embryonic stem cells and their neural derivatives, determined using flow-cytometric analysis, reveal a novel CD marker for exclusion of pluripotent stem cells', *Stem Cell Research*, 2(2), pp. 113–124. doi: 10.1016/j.scr.2008.08.001.

Suter, D. M. and Krause, K. H. (2008) 'Neural commitment of embryonic stem cells: Molecules,

pathways and potential for cell therapy', *Journal of Pathology*, pp. 355–368. doi: 10.1002/path.2380.

Suter, D. M., Tirefort, D., Julien, S. and Krause, K.-H. (2009) 'A Sox1 to Pax6 switch drives neuroectoderm to radial glia progression during differentiation of mouse embryonic stem cells.', *Stem cells (Dayton, Ohio)*, 27(1), pp. 49–58. doi: 10.1634/stemcells.2008-0319.

Tachibana, M., Amato, P., Sparman, M., Gutierrez, N. M., Tippner-Hedges, R., Ma, H., Kang, E., Fulati, A., Lee, H. S., Sritanaudomchai, H., Masterson, K., Larson, J., Eaton, D., Sadler-Fredd, K., Battaglia, D., Lee, D., Wu, D., Jensen, J., Patton, P., Gokhale, S., Stouffer, R. L., Wolf, D. and Mitalipov, S. (2013) 'Human embryonic stem cells derived by somatic cell nuclear transfer', *Cell*, 153(6), pp. 1228–1238. doi: 10.1016/j.cell.2013.05.006.

Takahashi, K. and Yamanaka, S. (2006) 'Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors', *Cell*, 126(4), pp. 663–676. doi: 10.1016/j.cell.2006.07.024.

Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T. and Akira, S. (1997) 'Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality.', *Proceedings of the National Academy of Sciences of the United States of America*, 94(8), pp. 3801–4. doi: 10.1073/pnas.94.8.3801.

Táncos, Z., Nemes, C., Varga, E., Bock, I., Rungarunlert, S., Tharasanit, T., Techakumphu, M., Kobolák, J. and Dinnyés, A. (2017) 'Establishment of a rabbit induced pluripotent stem cell (RbiPSC) line using lentiviral delivery of human pluripotency factors', *Stem Cell Research*, pp. 16–18. doi: 10.1016/j.scr.2017.03.010.

Tang, K., Peng, G., Qiao, Y., Song, L. and Jing, N. (2015) 'Intrinsic regulations in neural fate commitment', *Development Growth and Differentiation*, pp. 109–120. doi: 10.1111/dgd.12204.

Thomson, J., Itskovitz-Eldor, J., Shapiro, S. S. S., Waknitz, M. A. A., Swiergiel JJ, Marshall, V. S. S. and Jones, J. M. (1998) 'Embryonic Stem Cell Lines Derived from Human Blastocysts', *Science*, 282(5391), pp. 1145–1147. doi: 10.1126/science.282.5391.1145.

Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., Becker, R. A. and Hearn, J. P. (1995) 'Isolation of a primate embryonic stem cell line.', *Proceedings of the National Academy of Sciences of the United States of America*, 92(17), pp. 7844–7848. doi: PMC41242.

Thomson, J. A. and Odorico, J. S. (2000) 'Human embryonic stem cell and embryonic germ cell lines', *Trends in Biotechnology*, 18(2), pp. 53–57. doi: 10.1016/S0167-7799(99)01410-9.

Till, J. E. and McCulloch, E. (1961) 'A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. 1961.', *Radiation research*, 175(2), pp. 145–149. doi: 10.1667/RRXX28.1.

Tokumoto, Y., Ogawa, S., Nagamune, T. and Miyake, J. (2010) 'Comparison of efficiency of terminal differentiation of oligodendrocytes from induced pluripotent stem cells versus embryonic stem cells in vitro.', *Journal of bioscience and bioengineering*, 109(6), pp. 622–8. doi: 10.1016/j.jbiosc.2009.11.013.

Tucker, B. A., Park, I.-H., Qi, S. D., Klassen, H. J., Jiang, C., Yao, J., Redenti, S., Daley, G. Q. and Young, M. J. (2011) 'Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice.', *PloS one*, 6(4), p. e18992. doi: 10.1371/journal.pone.0018992.

Turnpenny, L., Brickwood, S., Spalluto, C. M., Piper, K., Cameron, I. T., Wilson, D. I. and Hanley, N. A. (2003) 'Derivation of Human Embryonic Germ Cells: An Alternative Source of Pluripotent Stem Cells', *Stem Cells*. John Wiley & Sons, Ltd., 21(5), pp. 598–609. doi:

10.1634/stemcells.21-5-598.

Ueberham, U. and Arendt, T. (2013) ‘The Role of Smad Proteins for Development, Differentiation and Dedifferentiation of Neurons’, in *Trends in Cell Signaling Pathways in Neuronal Fate Decision*. InTech. doi: 10.5772/54532.

Ullian, E. M., Sapperstein, S. K., Christopherson, K. S. and Barres, B. a (2001) ‘Control of synapse number by glia.’, *Science (New York, N.Y.)*, 291(5504), pp. 657–661. doi: 10.1126/science.291.5504.657.

Valenta, T., Hausmann, G. and Basler, K. (2012) ‘The many faces and functions of β-catenin’, *The EMBO Journal*, 31(12), pp. 2714–2736. doi: 10.1038/emboj.2012.150.

Verlinsky, Y., Strelchenko, N., Kukharenko, V., Rechitsky, S., Verlinsky, O., Galat, V. and Kuliev, a (2005) ‘Human embryonic stem cell lines with genetic disorders.’, *Reproductive biomedicine online*, 10(I), pp. 105–110. doi: 10.1016/S1472-6483(10)60810-3.

Vogt, J., Traynor, R. and Sapkota, G. P. (2011) ‘The specificities of small molecule inhibitors of the TGFß and BMP pathways’, *Cellular Signalling*, 23(11), pp. 1831–1842. doi: 10.1016/j.cellsig.2011.06.019.

Waddington, C. (1956) ‘Genetic Assimilation of the Bithorax Phenotype’, *Evolution*, 10(1), pp. 1–13. doi: 10.2307/2406091.

Waddington, C. H. (1957) ‘The strategy of the genes. A discussion of some aspects of theoretical biology. With an appendix by H. Kacser.’, *The strategy of the genes A discussion of some …*, p. ix +-262 . doi: 10.1007/3-540-32786-X_7.

Wakazono, T., Yamashiro, K., Oishi, A., Ooto, S., Tamura, H., Akagi-Kurashige, Y., Hata, M., Takahashi, A., Tsujikawa, A. and Yoshimura, N. (2017) ‘Recurrence Of Choroidal Neovascularization Lesion Activity After Aflibercept Treatment For Age-Related Macular Degeneration’, *Retina*, 13(1), pp. 1–10. doi: 10.1097/IAE.0000000000001451.

Wang, L., Colodner, K. J. and Feany, M. B. (2011) ‘Protein misfolding and oxidative stress promote glial-mediated neurodegeneration in an Alexander disease model.’, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 31(8), pp. 2868–77. doi: 10.1523/JNEUROSCI.3410-10.2011.

Wang, Y. and Qin, Z.-H. (2010) ‘Molecular and cellular mechanisms of excitotoxic neuronal death.’, *Apoptosis : an international journal on programmed cell death*, 15(11), pp. 1382–1402. doi: 10.1007/s10495-010-0481-0.

Warmflash, A., Sorre, B., Etoc, F., Siggia, E. D. and Brivanlou, A. H. (2014) ‘A method to recapitulate early embryonic spatial patterning in human embryonic stem cells.’, *Nature methods*, 11(8), pp. 847–54. doi: 10.1038/nmeth.3016.

Wegner, M. (2011) ‘SOX after SOX: SOXession regulates neurogenesis’, *Genes and Development*, 25(23), pp. 2423–2428. doi: 10.1101/gad.181487.111.

Weiss, N., Miller, F., Cazaubon, S. and Couraud, P. O. (2009) ‘The blood-brain barrier in brain homeostasis and neurological diseases’, *Biochimica et Biophysica Acta - Biomembranes*, pp. 842–857. doi: 10.1016/j.bbamem.2008.10.022.

Wichterle, H., Lieberam, I., Porter, J. A. and Jessell, T. M. (2002) ‘Directed differentiation of embryonic stem cells into motor neurons’, *Cell*, 110(3), pp. 385–397. doi: 10.1016/S0092-8674(02)00835-8.

William, R. and Burch, R. (1959) *The Principles of Humane Experimental Technique by W.M.S. Russell and R.L. Burch*. Available at: http://altweb.jhsph.edu/pubs/books/humane_exp/het-toc (Accessed: 5 January 2017).

Wilmut, I., Bai, Y. and Taylor, J. (2015) 'Somatic cell nuclear transfer: origins, the present position and future opportunities.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 370(1680), p. 20140366. doi: 10.1098/rstb.2014.0366.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. and Campbell, K. H. (1997) 'Viable offspring derived from fetal and adult mammalian cells', *Nature*, 385(6619), pp. 810–813. doi: 10.1038/385810a0.

Wingerchuk, D. M., Lennon, V. A., Lucchinetti, C. F., Pittock, S. J. and Weinshenker, B. G. (2007) 'The spectrum of neuromyelitis optica.', *The Lancet. Neurology*, 6(9), pp. 805–15. doi: 10.1016/S1474-4422(07)70216-8.

Yagi, T., Ito, D., Okada, Y., Akamatsu, W., Nihei, Y., Okano, H. and Suzuki, N. (2012) 'Modeling familial Alzheimer's disease with induced pluripotent stem cells.', *Rinshō shinkeigaku = Clinical neurology*, 52(11), pp. 1134–6. doi: 10.1093/hmg/ddr394.

Yamanaka, S. and Blau, H. M. (2010) 'Nuclear reprogramming to a pluripotent state by three approaches.', *Nature*, 465(7299), pp. 704–12. doi: 10.1038/nature09229.

Ying, Q.-L., Stavridis, M., Griffiths, D., Li, M. and Smith, A. (2003) *Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture.*, *Nature biotechnology*. doi: 10.1038/nbt780.

Ying, Q.-L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P. and Smith, A. (2008) 'The ground state of embryonic stem cell self-renewal.', *Nature*, 453(7194), pp. 519–523. doi: 10.1038/nature06968.

Ying, Q.-L., Ying, Q.-L., Wray, J., Wray, J., Nichols, J., Nichols, J., Batlle-Morera, L., Batlle-Morera, L., Doble, B., Doble, B., Woodgett, J., Woodgett, J., Cohen, P., Cohen, P., Smith, A. and Smith, A. (2008) 'The ground state of embryonic stem cell self-renewal.', *Nature*, 453(May), pp. 519–23. doi: 10.1038/nature06968.

Yoo, S. J., Kim, J., Lee, C.-S. and Nam, Y. (2011) 'Simple and novel three dimensional neuronal cell culture using a micro mesh scaffold.', *Experimental neurobiology*. Korean Society for Brain and Neural Science, 20(2), pp. 110–5. doi: 10.5607/en.2011.20.2.110.

Yoshida, T., Ozawa, Y., Suzuki, K., Yuki, K., Ohyama, M., Akamatsu, W., Matsuzaki, Y., Shimmura, S., Mitani, K., Tsubota, K. and Okano, H. (2014) 'The use of induced pluripotent stem cells to reveal pathogenic gene mutations and explore treatments for retinitis pigmentosa.', *Molecular brain*, 7, p. 45. doi: 10.1186/1756-6606-7-45.

Yuan, T., Liao, W., Feng, N.-H., Lou, Y.-L., Niu, X., Zhang, A.-J., Wang, Y. and Deng, Z.-F. (2013) 'Human induced pluripotent stem cell-derived neural stem cells survive, migrate, differentiate, and improve neurological function in a rat model of middle cerebral artery occlusion.', *Stem Cell Research & Therapy*, 4(3), p. 73. doi: 10.1186/scrt224.

Zagoura, D., Canovas-Jorda, D., Pistollato, F., Bremer-Hoffmann, S. and Bal-Price, A. (2016) 'Evaluation of the rotenone-induced activation of the Nrf2 pathway in a neuronal model derived from human induced pluripotent stem cells', *Neurochemistry International*. doi: 10.1016/j.neuint.2016.09.004.

Zeng, H., Guo, M., Martins-Taylor, K., Wang, X., Zhang, Z., Park, J. W., Zhan, S., Kronenberg, M. S., Lichtler, A., Liu, H. X., Chen, F. P., Yue, L., Li, X. J. and Xu, R. H. (2010) 'Specification of region-specific neurons including forebrain glutamatergic neurons from human induced pluripotent stem cells', *PLoS ONE*, 5(7). doi: 10.1371/journal.pone.0011853.

Zhang, S. C., Wernig, M., Duncan, I. D., Brüstle, O. and Thomson, J. a (2001) 'In vitro differentiation of transplantable neural precursors from human embryonic stem cells.', *Nature biotechnology*, 19(12), pp. 1129–1133. doi: 10.1038/nbt1201-1129.

Zhang, X., Huang, C. T., Chen, J., Pankratz, M. T., Xi, J., Li, J., Yang, Y., LaVaute, T. M., Li, X. J., Ayala, M., Bondarenko, G. I., Du, Z. W., Jin, Y., Golos, T. G. and Zhang, S. C. (2010a) 'Pax6 is a human neuroectoderm cell fate determinant', *Cell Stem Cell*, 7(1), pp. 90–100. doi: 10.1016/j.stem.2010.04.017.

Zhang, X., Huang, C. T., Chen, J., Pankratz, M. T., Xi, J., Li, J., Yang, Y., LaVaute, T. M., Li, X. J., Ayala, M., Bondarenko, G. I., Du, Z. W., Jin, Y., Golos, T. G. and Zhang, S. C. (2010b) 'Pax6 is a human neuroectoderm cell fate determinant', *Cell Stem Cell*, 7(1), pp. 90–100. doi: 10.1016/j.stem.2010.04.017.

Zhang, Y., Sloan, S. A., Clarke, L. E., Caneda, C., Plaza, C. A., Blumenthal, P. D., Vogel, H., Steinberg, G. K., Edwards, M. S. B., Li, G., Duncan, J. A., Cheshier, S. H., Shuer, L. M., Chang, E. F., Grant, G. A., Gephart, M. G. H. and Barres, B. A. (2016) 'Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse', *Neuron*, 89(1), pp. 37–53. doi: 10.1016/j.neuron.2015.11.013.

Zhang, Z., Liao, B., Xu, M. and Jin, Y. (2007) 'Post-translational modification of POU domain transcription factor Oct-4 by SUMO-1.', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 21(12), pp. 3042–51. doi: 10.1096/fj.06-6914com.

Zhou, S., Szczesna, K., Ochalek, A., Kobolk, J., Varga, E., Nemes, C., Chandrasekaran, A., Rasmussen, M., Cirera, S., Hyttel, P., Dinnyes, A., Freude, K. K. and Avci, H. X. (2016) 'Neurosphere based differentiation of human IPSC improves astrocyte differentiation', *Stem Cells International*, 2016. doi: 10.1155/2016/4937689.

Zhou, W. and Freed, C. R. (2009) 'Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells', *Stem Cells*, 27(11), pp. 2667–2674. doi: 10.1002/stem.201.

Zhou, Y. and Zeng, F. (2013) 'Integration-free Methods for Generating Induced Pluripotent Stem Cells', *Genomics, Proteomics & Bioinformatics*, 11(5), pp. 284–287. doi: 10.1016/j.gpb.2013.09.008.

Zirra, A., Wiethoff, S., Patani, R., Zirra, A., Wiethoff, S. and Patani, R. (2016) 'Neural Conversion and Patterning of Human Pluripotent Stem Cells: A Developmental Perspective', *Stem Cells International*. Hindawi Publishing Corporation, 2016(1), pp. 1–14. doi: 10.1155/2016/8291260.

Zonta, M., Angulo, M. C., Gobbo, S., Rosengarten, B., Hossmann, K.-A., Pozzan, T. and Carmignoto, G. (2003) 'Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation.', *Nature neuroscience*, 6(1), pp. 43–50. doi: 10.1038/nn980.

Zwaka, T. P. and Thomson, J. a (2003) 'Homologous recombination in human embryonic stem cells.', *Nature biotechnology*, 21(3), pp. 319–21. doi: 10.1038/nbt788.

10.14751/SZIE.2017.064

10.14751/SZIE.2017.064

PUBLICATION LIST RESULTING FROM THE DOCTORAL WORK

International paper publications:

- **Chandrasekaran, A.,** Avci, H. X., Leist, M., Kobolák, J., and Dinnyés, A. (2016a). Astrocyte Differentiation of Human Pluripotent Stem Cells: New Tools for Neurological Disorder Research. *Front. Cell. Neurosci.* 10, 215. doi:10.3389/fncel.2016.00215. Impact Factor: 4.608.
- **Chandrasekaran, A.,** Varga, E., Nemes, C., Táncos, Z., Kobolák, J., and Dinnyés, A. (2016b). Establishment of induced pluripotent stem cell (iPSC) line from a 63-year old patient with late onset Alzheimer's disease (LOAD). *Stem Cell Res.* 17, 78–80. doi:10.1016/j.scr.2016.05.014. Impact Factor: 3.892.
- Zhou, S., Szczesna, K., Ochalek, A., Kobolak, J., Varga, E., Nemes, C., **Chandrasekaran, A.,** Rasmussen, M., Cirera, S., Hyttel, P., Dinnyés, A., Freude, K. K. and Avci, H. X. (2016). Neurosphere based differentiation of human IPSC improves astrocyte differentiation. *Stem Cells Int.* 2016. doi:10.1155/2016/4937689. Impact Factor: 4.10.
- Poon, A., Zhang, Y., **Chandrasekaran, A**., Phanthong, P., Schmid, B., Nielsen, T., Freude, K. (2017) Modeling neurodegenerative diseases with patient-derived induced pluripotent cells: possibilities and challenges. *New Biotechnology*. pii: S1871-6784(16)32499-2. doi: 10.1016/j.nbt.2017.05.009. Impact Factor: 3.813.
- Nagy, J., Kobolak, J., Berzsenyi, S., Abraham, Z., Avci, H., Bock, I., Bekes, Z., Hodoscsek, B., **Chandrasekaran, A**., Téglási, A., Dezso, P., Kovanyi, B., Tarnoke, E., Fodor, L., Szel, T., Nemeth, K., Balaza, A., Dinnyés, A., Lendvai, B., Levay, G and Roman, V.Altered neurite morphology and cholinergic function of induced pluripotent stem cell-derived neurons from a patient with Kleefstra syndrome and autism. Transl Psychiatry 2017 Jul 25;7(7):e1179. Epub 2017 Jul 25. doi:10.1038/tp.2017.144. Impact factor: 4.73

Publications in preparation:

- **Chandrasekaran, A,** Avci, HX, Ochalek, A, Rösingh, LN, Molnár, K, László, L, Bellák, T, Téglási, A, Pesti, K, Mike, A, Phanthong, Hall, V, Kitiyanant, N, Krause, KH, Kobolák, J and Dinnyés A (2017) Comparison of 2D and 3D neural induction methods for the generation of neural progenitor cells from human iPSCs. Manuscript under revision; *Stem Cell Research*; number: SCR-S-17-00155.
- Ochalek, A; Mihalik, B; Avci, HA; **Chandrasekaran, A**; Téglási, A; Bock, I; Giudice, ML; Táncos, Z; Molnár, K; László, L; Nielsen, JE; Holst, B; Freude, K; Hyttel, P; Kobolák, J; Dinnyes, A. (2017). Neurons derived from sporadic Alzheimer's disease iPSCs reveal elevated TAU hyperphosphorylation, increased amyloid levels and GSK3B activation. Manuscript submitted; *Molecular Neurodegeneration*; number: MOND-D-17-00196.

International abstract and poster presentations:

- **Chandrasekaran** A, Roesingh LN, Ochalek A, Nemes C, Varga E, Bock I, Zhou S, Szegedi V, Avci H, H. Raveh-Amit, Kobolák J, A. Dinnyes. Neuronal differentiation

from human induced pluripotent stem cells in IDPbyNMR Final meeting. Grosseto, Italy, 21-26. September 2014.

- **Chandrasekaran A**, Roesingh LN, Ochalek A, Nemes C, Varga E, Bock I, Avci H, Kobolak J, A. Dinnyes. Neuronal differentiation of patient specific induced pluripotent stem cells in Stem cells in cancer and regenerative medicine EMBO Conference. 2014. 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.
- Ochalek A, Bellák T, **Chandrasekaran A,** Szegedi V, Bock I, Varga E, Nemes C, Zhou S, Hyttel P, Kobolák J, Dinnyés A, Aci H. Engineering high and low complex 3D neural tissue from human induced pluripotent stem cells. Stem Cell Models of Neural Regeneration and Disease Dresden, Germany 1-3 February 2016.
- Roman V, Kobolák J, Avci H, Ábrahám Z, Hodoscsek B, Berzsenyi S, Koványi B, Dezső P, Nagy J, **Chandrasekaran A,** Ochalek A, Varga E, Nemes C, Bock I, Pentelényi K, Németh K, Balázs A, Molnár J, Dinnyés A, Lévay G, Lendvai B Compromised Neurite Morphology of Induced Pluripotent Stem Cell-Derived Neurons: Similar Patterns from Independent Non-Syndromic Autism Cases. 2016 International Meeting for Autism Research (IMFAR) Baltimore, MD, USA 11-14 May 2016
- Francz B, Schmidt B, Ochalek A, **Chandrasekaran A**, Kovács E, Mihalik B, Kobolák J, Dinnyés *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:

- **Chandrasekaran A**, Roesingh LN, Berzsenyi S, Kobolak J, Dinnyes A. Small molecule induced neuronal differentiation of hiPS cell lines through NPC derivation "Fiatal Biotechnológusok Országos Konferenciája (FIBOK). 2014. Young Biotechnologists' National Conference Szeged, Hungary. 7 March 2014.
- **Chandrasekaran A**, Roesingh LN, Ochalek A, Nemes C, Varga E, Bock I, Zhou S, Szegedi V, Avci H, Kobolak J, A. Dinnyes Patient specific induced pluripotent stem cells and their neuronal differentiation in Annual Meeting if the Hungarian Biochemical Society. Debrecen, Hungary, August 2014 2014. p. 36.
- **Chandrasekaran A**, Roesingh LN, Ochalek A, Nemes C, Varga E, Bock I, Szczesna K Avci H, Kobolak J, A. Dinnyes. Comparison of 2D and 3D neuronal differentiation of patient specific induced pluripotent stem cells "XV. Biannual Conference of the Hungarian Neuroscience Society" Hungarian Academy of Sciences Budapest, Hungary. 22-23 January 2015.

- Bellák.T, **Chandrasekaran A** , Ochalek A, Szegedi V, Varga E, Nemes C, Zhou S, Avci H, Kobolak J, Dinnyes A. 3D engineered neural tissue from human induced pluripotent stem cells “XV. Biannual Conference of the Hungarian Neuroscience Society” Hungarian Academy of Sciences Budapest, Hungary. 22-23 January 2015.
- Bellák.T, **Chandrasekaran A** , Ochalek A, Szegedi V, Varga E, Nemes C, Zhou S, Avci H, Kobolak J, Dinnyes A: 3D engineered neural tissue from human induced pluripotent stem cells. In “Hungarian Molecular Life Sciences 2015″ Conference in Eger, Hungary 27-29 March 2015.
- Bellák T, Ochalek A, **Chandrasekaran A**, Szegedi V, Avci H, Szczesna K, Varga E, Nemes C, Nógrádi A, Kobolák J, Dinnyés A. Modelling Alzheimer’s Disease with three-dimensional engineered neural tissue. 19th Congress of the Hungarian Anatomical Society, Szeged, Hungary 11-13 June 2015
- Ochalek A, **Chandrasekaran A**, Bellák T, Avci H, Szegedi V, Varga E, Nemes C, Schmidt B, Bock I, Zhou S, Szczesna K, Kobolák J, Dinnyés A 3D engineered neural tissue from patient-derived induced pluripotent human induced pluripotent stem cells as a tool for modelling Alzheimer disease. HuNDoC 2016 Budapest, Hungary 20 January 2016.
- Francz B, Schmidt B, Ochalek A, **Chandrasekaran A**, Kovács E, Mihalik B, Kobolák J, Dinnyés A Induced Pluripotent Stem cell-based *in vitro* neurotoxicology models HuNDoC 2016 Budapest, Hungary 20 January 2016.
- Ochalek A, **Chandrasekaran A**, Bellák T, Avci H, Szegedi V, Varga E, Nemes C, Schmidt B, Bock I, Zhou S, Szczesna K, Kobolák J, Dinnyés A 3D engineered neural tissue from patient-derived induced pluripotent human induced pluripotent stem cells as a tool for modelling Alzheimer disease. IBRO Workshop Hungarian Academy of Sciences Budapest, Hungary 21-22 January 2016.
- Francz B, Schmidt B, Ochalek A, **Chandrasekaran A**, Kovács E, Mihalik B, Kobolák J, Dinnyés A Induced Pluripotent Stem cell-based *in vitro* neurotoxicology models Fiatal Biotechnológusok Országos Konferenciája (FIBOK) Godollo, Hungary 21-22 March 2016.
- Chandrasekaran, A, Avci, HX, Ochalek, A, Molnár, K, László, L, Bellák, T, Pesti, K, Mike, A, Kobolák, J and Dinnyés A: Comparison of 2D and 3D neural induction methods for the generation of neural progenitor cells (NPCs) from human iPSCs. Hungarian Molecular Life Sciences 2017; Eger, Hungary; March, 31st - April 2nd, 2017.

Oral Presentation:

- **Chandrasekaran A** presented “Presenilin 1/2 and their mutation in Alzheimer Disease” Alzheimer Workshop, Biotalentum, Hungary. 9th September 2014.
- **Chandrasekaran A** , Roesingh LN, Nemes C, Varga E, Bock I, Zhou S, Ochalek A, Szegedi V, Avci H, Dinnyes A, Kobolak J. (2014). Induced ploripotent stem cells for biomedical applications. Innovations in Medicine, Budapest, Hungary.p. 5, 10 October, 2014

- **Chandrasekaran A**, Neelchen RL, Nemes C, Varga E, Bock I, Zhou S, Szegedi V, Avci H, Dinnyes A, Kobolak J. (2015). Neuronal differentiation of human patient specific induced pluripotent stem cells. Lecture at GlowBrain III. Workshop, Visualisation of molecular markers in the brain, Zagreb, Croatia. 29-31, January, 2015
- Kobolák J, **Chandrasekaran A**, Ochalek A, Bellák T, Szegedi V,Varga E, Avci H, Zhou S, Szczesna K, Smidth B, Dinnyés A: Patient specific induced pluripotent stem cells (iPSCs) and their neuronal differentiation in Alzheimer's disease. In "Hungarian Molecular Life Sciences 2015″ Conference in Eger, Hungary 27-29 March 2015.
- Dinnyes A, **Chandrasekaran A**, Ochalek A, Bellák T ,Szegedi V, Avci H, Eszter V, Téglási A, Eszter K, Szczesna K, Schmidt B, Kobolák J (2015). "Betegspecifikus őssejtek differenciójának neurális és kardiális irányokba." Lecture at Hungarian experimental and clinical pharmacological company's experimental pharmacological IX. symposium, Velence, Hungary. p. 41, 26-28, March, 2015
- Szczesna K, **Chandrasekaran, A**, A. Dinnyes,"Nanomaterials interacting with biological barriers, with special emphasis on blood brain barrier crossing molecules, an important tool to treat/diagnose neurodegenerative diseases" MCN ITN-NanoS3, Hotel Erzsébet City Center, and Hungary.23th April 2015.
- **Chandrasekaran A** Patient specific induced pluripotent stem cells (iPSCs) and their neuronal differentiation Utrecht University, Netherlands. 13th June 2016.

APPENDICES

Appendix A:

Supplementary Table 1. Antibodies used for immunocytochemistry

	Antibody	Dilution	Company (Cat #)
Pluripotency	mouse anti-E-CADHERIN	1:1000	Thermo Fisher Scientific Inc. (13-1700)
	mouse anti-OCT4	1:50	Santa Cruz Biotechnologies (sc-5279)
	goat anti-hNANOG	1:50	R&D (AF1997)
***in vitro* Differentiation**	mouse anti-NESTIN	1:1000	Millipore (MAB5326)
	rabbit anti-ß-III-TUBULIN	1:2000	Covance (PRB-435P)
	rabbit anti-BRACHYURY	1:50	Santa Cruz Biotechnologies (sc-20109)
	mouse anti-GATA4	1:50	Santa Cruz Biotechnologies (sc-25310)
Neuronal differentiation	mouse anti-hNESTIN	1:1000	Millipore (MAB5326)
	rabbit anti-PAX6	1:500	Covance (PRB278P)
	goat anti-SOX1	1:50	R&D (AF3369)
	rabbit anti-MAP2	1:1000	Millipore (MAB3418)
	rabbit anti-ß-III-TUBULIN	1:1000	Covance (PRB-435P)
	mouse anti-ß-III-TUBULIN	1:1000	Santa Cruz (SC-58888)
	goat anti-GFAP	1:50	Santa Cruz (C-19 SC)
	rabbit anti-GFAP	1:1000	Dako (Z0334)
	rabbit anti-aquaporin4	1:50	Santa Cruz (SC20812)
	mouse anti- CUX1	1:1000	Abcam (AB5483)
	rabbit anti- TBR1	1:250	Abcam (AB31940)
Secondary Antibodies	Alexa Fluor 488 donkey anti-goat IgG	1:2000	Thermo Fisher Scientific Inc. (A-11055)
	Alexa Fluor 488 donkey anti-mouse IgG	1:2000	Thermo Fisher Scientific Inc. (A-21202)
	Alexa Fluor 488 donkey anti-rabbit IgG	1:2000	Thermo Fisher Scientific Inc. (A-21206)
	Alexa Fluor 488 donkey anti-mouse IgM	1:2000	Thermo Fisher Scientific Inc. (A-21042)
	Alexa Fluor 488 donkey anti-mouse IgM	1:200	Jackson ImmunoResearch (715-545-020)
	Alexa Fluor 488 goat anti-rabbit IgG	1:3000	Thermo Fisher Scientific Inc. (A11008)
	Alexa Fluor 594 donkey anti-rabbit IgG	1:3000	Thermo Fisher Scientific Inc. (A10039)
	Alexa Fluor 594 donkey anti-goat IgG	1:3000	Thermo Fisher Scientific Inc. (A11058)
	Alexa Fluor 350 donkey anti-mouse IgG	1:3000	Thermo Fisher Scientific Inc. (A21203)
Conjugated Antibodies	PE-mouse anti-human PAX6	1:20	BD Bioscience (561552)
	Alexa Fluor 647 mouse anti-NESTIN	1:5	BD Bioscience (560393)

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Supplementary Table 2. Gene-specific Mouse and Human primers used for qRT-PCR analysis.

Mouse Primers	Forward Sequence	Reverse Sequence	Accession Number
Gapdh	AATGTGTCCGTCGTGGATCT	CCTGCTTCACCACCTTCTTG	NM_008084.2
Sox1	TCTGTATCCGAGCATTTCCA	CAACATCCGACTCCTCTTCC	NM_009233.3
Pax6	GGACCCATTATCCAGATGTGTT	TGGCCTGTCTTCTCTGGTTC	NM_013627.5
Nestin	AAGAAGAACCAAGAATGGAGGA	CTGTCTCTAGTGTGTGTTCAGGAGA	NM_016701.3
Human Primers	**Forward Sequence**	**Reverse Sequence**	**Accession Number**
GAPDH	CTCTCTGCTCCTCCTGTTCGAC	TGAGCGATGTGGCTCGGCT	NM_002046.5
B2M	GTGCTCGCGCTACTCTCTCT	TCAATGTCGGATGGATGAAA	NM_004048.2
SOX1	TAGTAAGGCAGGTCCAAGCA	GGGTGGTGGTGGTAATCTCT	NM_005986.2
PAX6	GCCAGCAACACACCTAGTCA	TGTGAGGGCTGTGTCTGTTC	NC_000011.10
NESTIN	ACTGAAGTCTGCGGGACAAG	CAGTGGTGCTTGAGTTTCTG	NM_006617.1
TUBB3	AACGAGGCCTCTTCTCACAA	GGCCTGAAGAGATGTCCAAA	NM_001197181.1
MAP2	TTGTCTCTAACCGAGGAAGCA	TCGTTGTGTCGTGTTCTCAA	NM_031847.2

Supplementary Table 3. Statistical data

	Cell line	2D (Mean ± SEM)	3D (Mean ± SEM)	P value
Nestin+/ Pax6+	CL-1	83.7±0.77 %	94.2±0.74 %	p < 0.05
	CL-2	85.4±0.55 %	93.5±0.78 %	p < 0.05
	DL-1	93.2±1.50 %	97.4±0.44 %	p < 0.05
	DL-2	86.3±0.46 %	94.8±1.30 %	p < 0.05
	DL-3	81.9±0.79 %	94.3±0.74 %	p < 0.05
SOX1+	CL-1	77.3±1.27 %	81.7±1.50 %	p < 0.05
	CL-2	80.0±2.26 %	75.5±1.08 %	p < 0.05
	DL-1	79.3±2.58 %	71.2±1.19 %	p < 0.05
	DL-2	83.0±2.72 %	76.1±3.81 %	p < 0.05
	DL-3	81.9±1.80 %	76.5±3.51 %	p < 0.05
SOX9+	CL-1	10.2±0.85 %	9.7±1.00 %	ns
	CL-2	11.4±0.27 %	12.8±1.03 %	ns
	DL-1	9.1±1.15 %	13.0±1.11 %	p < 0.05
	DL-2	8.9±0.90 %	6.5±0.79 %	p < 0.05
	DL-3	12.2±1.46 %	10.6±1.21 %	ns
TBR1+	CL-1	9.3±0.93 %	16.4±4.55 %	p < 0.05
	CL-2	11.3±2.80 %	22.9.1±4.9 %	p < 0.05
	DL-1	12.9±1.96 %	17.6±1.34 %	ns
	DL-2	22.4±2.20 %	11.2±1.16 %	p < 0.05
	DL-3	20.2±2.80 %	7.0±0.96 %	p < 0.05
CUX1+	CL-1	12.5±1.78 %	19.0±5.60 %	p < 0.05
	CL-2	10.6±1.69 %	12.0±1.36 %	ns
	DL-1	11.3±1.72 %	15.7±1.33 %	ns
	DL-2	13.5±0.64 %	13.2±2.93 %	ns
	DL-3	14.2±1.15 %	7.2±0.86 %	p < 0.05
GFAP+	CL-1	28.1±1.86 %	31.7±1.51 %	ns
	CL-2	45.3±1.69 %	48.1±11.41 %	p < 0.05
	DL-1	3.12±0.12 %	5.3±0.75 %	ns
	DL-2	7.1±0.62 %	16.7±5.74 %	p < 0.05
	DL-3	35.7±2.04 %	40.0±5.38 %	p < 0.05
AQP4+	CL-1	17.7±2.01 %	20.6±1.07 %	ns
	CL-2	30.5±1.70 %	6.6±1.90 %	p < 0.05
	DL-1	1.0±0.23 %	7.7±2.10 %	p < 0.05
	DL-2	1.8±0.27 %	6.8±0.86 %	p < 0.05
	DL-3	36.4±3.00 %	32.2±4.74 %	p < 0.05
Neuronal Length	CL-1	213.4±13.36 µm	258.2±12.53 µm	p < 0.05
	CL-2	261.9±35.94 µm	401.9±90.80 µm	p < 0.05
	DL-1	214.9±21.19 µm	236.8±12.71 µm	ns
	DL-2	367.2±26.52 µm	461.2±25.21 µm	p < 0.05
	DL-3	208.5±32.72 µm	287.1±15.66 µm	p < 0.05

Statistical analysis of readouts comparing the 2D/3D neuronal induction culture conditions. P indicates p <0.05.

Appendix B: Media compositions

1. Mouse Thawing Medium

Reagent	End Concentration
Mouse ESC media	100 %
Rock inhibitor (10mM)	10µM

2. Mouse Embryonic Stem Cell (mESC) Medium

Reagent	End Concentration
GMEM or DMEM/F12 (d-(+)-glucose)	50 %
FBS	10%
Sodium - Pyruvate	2 mM
Glutamax	2 mM
MEM NEAA	100µM
β-mercaptoethanol	50 µM
Pen/Strep	50 U/mL
LIF	1000 U/mL

3. Mouse Maintenance 2i Medium

Reagent	End Concentration
GMEM or DMEM/F12 (d-(+)-glucose)	50 %
FBS	10%
Sodium - Pyruvate	2 mM
Glutamax	2 mM
MEM NEAA	100µM
Insulin	5 mg/ml
PD0325901 (mitogen-activated protein kinase inhibitor)	1 µM
CHIR99021 (glycogen synthase kinase-3 inhibitor)	3 µM
β-mercaptoethanol (Optional)	50 µM

4. Mouse 2D Neuronal Induction Medium, N2B27 media

Reagent	End Concentration
DMEM/F12 (d-(+)-glucose)	50 %
Neurobasal medium	50 %
N-2 (100x)	1 %
B-27 (50x)	2 %
BSA	50 µg/ml
Glutamax	2 mM
Pen/Strep	50 U/mL
Insulin	7,5µg/mL
β-mercaptoethanol (	50 µM
EGF 100ug/ml	10ng/mL
bFGF 100ug/ml	10ng/mL

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5. Mouse Neuronal Differentiation Medium

Reagent	End Concentration
DMEM/F12 D-(+)-glucose	50%
Neurobasal medium	50 %
N-2 (100x)	0.5 %
B-27 (50x)	1 %
Glutamax	3 mg/mL
Pen/Strep	50 U/mL
β-mercaptoethanol	50 μM

6. Mouse Astrocyte Differentiation Medium

Reagent	End Concentration
mouse N2B27 media	100 %
BMP4	20ng/mL

7. Rat Astrocyte Differentiation Media-Thermo Fisher Ltd

Reagent	End Concentration
Dulbecco's Modified Eagle medium (high glucose)	85 %
Fetal Bovine Serum	15 %

8. Mouse and Human Freezing Medium

Reagent	Concentration	For 10 ml
KOSR (or) FBS	90 %	9 ml
DMSO	10 %	1 ml
Rock inhibitor (10mM)	End concentration10μM	

9. Human NPC Thawing Medium

Reagent	Concentration	For 10 ml
NMM media	100 %	10 ml
Rock inhibitor (10mM)	End concentration10μM	

10. Human 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 10μM	
*Noggin 100ug/ml	End concentration 500ng/mL	
*LDN193189 100ug/ml	End concentration 10ng/mL	
β-mercaptoethanol	End concentration 100μM	
Insulin	End concentration 5μg/ml	

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11. Human 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 25µM	

12. Human Neuronal Differentiation Medium (NDM)

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
Ascorbic acid	End concentration 0.2mM	
GDNF	End concentration 20ng/mL	
BDNF	End concentration 20ng/mL	
β-mercaptoethanol	End concentration 25µM	

13. Human Astrocyte Maintenance Medium (N2B27 media)

Reagent	Concentration
DMEM/F12 (d-(+)-glucose)	50 %
Neurobasal medium	50 %
N-2 (100x)	1 %
B-27 (50x)	2 %
BSA	End concentration 50 µg/ml
Glutamax	End concentration 1-2 mM
Pen/Strep	End concentration 50 U/mL
Insulin	End concentration 7,5µg/mL
EGF 100ug/ml	End concentration 10ng/mL
bFGF 100ug/ml	End concentration 10ng/mL
β-mercaptoethanol	End concentration 25µM

14. Human Astrocyte Differentiation Medium

Reagent	Concentration
N2B27 media ((-) bFGF and (-) EGF)	50 %
CNTF	End concentration 20ng/mL

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).

Cell lines	**References**
CTL-2	Zhou et al 2016
CTL-3	Zhou et al 2016
DL-1	Nemes *et al.*, 2016
DL-2	Chandrasekaran *et al.*, 2016
DL-3	Ochalek *et al.*, 2016

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PREFACE

This thesis is made as a completion of the Ph.D. education in Animal Husbandry. The Ph.D. project was carried out from 2013 (Aug) to 2017 (Jan) at Department of ***Animal Husbandry Science*** under the director of ***Prof. Dr. Miklós Mézes***, CMHAS, Szent Istvan University, Gödöllő, Hungary. The experimental part of the project was supported by the EU FP7 projects (**FP7-PEOPLE-2012-ITN 317146,** EpiHealthNet). The majority of the project was performed at Biotalentum Ltd., Gödöllő, Hungary, as an early stage Marie-curie researcher. Prof. Dr. András Dinnyés, DVM, Dsc, Szent Istvan University acted as the main supervisor and as co-supervisor Dr. Julianna Kobolák from Biotalentum, Gödöllő, Hungary. Postdoc Hasan X. Avci was involved in the planning and supervision of the experiments at Biotalentum Ltd., Gödöllő, Hungary.

This thesis entitled "***Differentiation of mouse and human Pluripotent Stem Cells*** "includes an introduction including the objectives of the study, a general background describing the relevant topics and techniques, summarized methods and materials, summarized results and discussions, conclusion, also perspectives. Finally, original manuscripts, which resulted from my Ph.D. project, are attached.