SZENT ISTVÁN UNIVERSITY

Animal Husbandry Science PhD School

NEURAL DIFFERENTIATION OF MOUSE AND HUMAN PLURIPOTENT STEM CELLS

The Thesis of the Ph.D. dissertation

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1. INTRODUCTION AND AIM

Small-animal models, like mouse and rat, offer apparent advantages regarding high reproductive rates, low maintenance costs and the ability to perform specific experiments. 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 translations 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 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. My current work specifies this translation in the field of neurobiology.

Neural progenitor cells (NPCs) are self-renewing and have the capacity to differentiate into neurons as well as glia (Breunig et al., 2011). There are many established protocols for generating pluripotent stem cell (PSC)-derived cortical projection neurons from both mouse (Gaspard et al., 2009) and human cells (Shi et al., 2012). However, efficient differentiation of these cells into specific neuronal subtypes remains challenging due to a large variation in outcomes following differentiation. This variation is likely due to the environment niche, heterogeneity in starting cell populations, differences in culture media and altered handling of cells (Engel et al., 2016). The crucial step in generating NPCs is to efficiently induce PSCs into early neuroepithelial progenitors (NEP) from the dorsal telencephalon (Chambers et al., 2009). Another prominent feature is the generation of neural "rosettes", which are morphologically identifiable structures containing neural progenitor cells, and which are believed to mimic the initial process of *in vivo* neural development (Wilson and Stice, 2006).

Over the years, NPCs derived from human PSCs have conventionally been induced using a 3D aggregate system, which is considered an efficient differentiation of PSCs into the neural lineage (Emdad et al., 2012). In

addition, studies revealed that neural induction in 3D micro fibrous matrices also resulted in increased expression of NPC markers as well as more mature neuron markers when compared to 2D neural induction (Liu et al., 2013). Despite these positive results, the 3D neural induction endure technical difficulties, such as, low efficiencies of cells forming 3D spheres, or difficulties in controlling the sphere's size and shape. In order to overcome these technical challenges, 2D neural induction was implemented to generate NPCs at a higher efficiency (Yan et al., 2013). Though Muratore et al., compared the commonly employed methods for neuronal differentiation from iPSCs but did not explain the initial neural induction changes (Muratore et al., 2014). Despite this overall progress, no publication has directly compared 3D neural induction (sphere) versus 2D neural induction (adherent, monolayer) which specifically analyse differences in cell numbers, gene expression profiles, proliferation rates, differentiation potential, cell fate or even functionality.

In the current study, we provide specific comparisons of these two systems following differentiation from different genetic background hiPSCs (healthy controls and patient-derived cell lines) into default neural fate through different induction systems. We identified that neural rosettes could be generated from both induction methods which later then acquired a cortical identity and differentiated into mature cortical neurons. However, we found that the 3D neural induction method yielded a higher number of NPCs and neurons with longer neurites. Electrophysiological studies did not reveal significant difference between the two different methods or cell lines, suggesting no change in function was observed.

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, can 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. MATERIALS AND METHODS

2.1 Mouse embryonic stem cell culture

The mouse embryonic stem cell (mESC) line HM1 was maintained in (i) CGR8 medium. Prior 2D neural induction the cells were transferred to feeder free condition where ESCs were cultured on gelatin-coated dishes in the presence of 2i inhibitors (CHIR99021-GSK inhibitor and PD184352-MERK inhibitor) to maintain the pluripotency (Ying et al., 2008; Nichols and Smith, 2009).

2.2 Induction of neuronal differentiation of mouse ESCs

2.2.1 2D Monolayer induction

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 () onto 10 cm bacteriological dishes precoated with 0.1% gelatin. Pluripotent cells were differentiated in N2B27 medium in monolayer for 6 Days. On Day 7, cells were plated onto Poly-ornithine, and Laminin (POL/L; 0.002%/1µg/cm2) coated flasks in N2B27medium containing (10 ng/mL) FGF2 and EGF.

2.2.2 Astrocyte differentiation of mouse ESCs

To promote astrocyte differentiation, cells were plated at a density of 30.000 cells/cm2. The astrocyte differentiation medium was changed every other day (for up to 5 days) containing 20 ng/ml BMP4 (Kleiderman et al., 2016).

2.2.4 Primary rat astrocyte culture

As an internal control, rat primary cortical astrocytes were purchased from Thermo Fisher Ltd. The rat astrocytes were maintained in uncoated; tissue culture treated flasks for maintenance and expansion. The cells were maintained in astrocyte growth medium (containing 85% DMEM /F12-high glucose and 15% FBS).

2.3 Human pluripotent stem cell culture

Human iPSCs used in this study were generated by Sendai virus-based reprogramming. 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) were used. All

the clones were maintained on BD Matrigel and mTESR1 media. Cultures were fed daily with mTESR1 and passaged every 5-7 Days for colony growth, following the instructions of the manufacturer.

2.4 Induction of neuronal differentiation of human iPSCs

2.4.1 Monolayer based (2D) neural induction

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). By Day 10, neural epithelial sheets were manually picked and re-plated onto POL/L plates. 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). 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.

2.4.2 Sphere based 3D neural induction

Likewise same SMAD molecues (SB431542 and Noggin) were used for 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. 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 containing 10 ng/mL of bFGF and EGF. Upon reaching confluence, NPCs were passaged and replated for terminal differentiation in a similar way of the 2D neuronal induction method.

2.4.3 Astrocyte differentiation of human PSCs

Astrocyte populations (NPCs over p9) were obtained by differentiating the late phase NPCs on POL/L coated plates or tissue culture flasks. Late NPC were plated in N2B27-medium containing 20 ng/ml ciliary neurotrophic

growth factor. The medium was changed every other day from Day 1, and experiments were performed for 35 Days.

2.5 Fluorescence activated cell sorting (FACS)

The NPCs were 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) for 1 hour at RT, while for the unconjugated primary antibodies isotype specific secondary antibodies were used accordingly. Cells were washed and analyzed using 'Flow Cytometer Cytomics FC 500' (Beckman Coulter).

2.6 Immunostaining

The NPCs were then incubated with the corresponding primary antibodies by overnight incubation at 4°C. On the next day; isotype specific secondary antibodies were diluted in blocking buffer and applied for 1 hour at RT. The cells were used accordingly to determine the signal. 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 immunostainings was performed as above, and was analyzed using FluoView FV10i confocal laser scanning microscope (Olympus Ltd, Tokyo, Japan).

2.7 Electron microscopy

The samples were fixed with a fixative solution for 24 hours on 4°C. Followed by fixative, 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).

2.8 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. The data was analyzed using REST software (2009 V2.0.13), and the statistics were analyzed using Graph Pad Prism 5.

2.9 Neurite length measurements

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 was 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 (Pool *et al.*, 2008).

2.10 Electrophysiological recordings

Whole cell recordings were carried out using an Axopatch 200 B amplifier and the pClamp software (Molecular Devices, Sunnyvale, CA). Voltage-gated ion channel-mediated currents were evoked by depolarizing pulses from a holding potential of -120 mV.

2.11 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).

3. RESULTS

3.1 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. 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.

The neuroectodermal differentiation was monitored in 48 hours intervals by gene expression profiling (qRT-PCR) and protein expression images (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. However, within 2 Days of neuronal differentiation, they subsequently express neuronal progenitor markers: Nestin, Sox1 and Pax6. Finally the neuronal population Tubb3 and Map2 was gradually increased from Day 6 onwards, thus leading to an efficient generation of NPCs.

The results of the neuronal expression on mRNA 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. We also 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, our 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.

3.2. 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 was effective in mouse studies, we set a direct comparison of 2D and 3D neural induction method in human iPSCs.

In our work, human blood samples were collected either from healthy volunteer individuals or diseased patients, with full consent. The generation procedure is published earlier by our team (Zhou *et al.*, 2016; Nemes *et al.*, 2016; Ochalek *et al.*, 2016; Táncos *et al.*, 2016).

The neural inductions were indistinguishable across the five different lines by gross morphological analysis. Neural rosettes were observed in both induction methods independent from the investigated hiPSC line either with phase contrast and confocal microscopy or with electron microscopy. 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. Moreover, we did not observe significant differences in the efficiency of spheroid formation among the different hiPSC lines.

Especially 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). The majority of the cells co-expressed NESTIN and PAX6. Especially 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 microscopy analysis we were able to observe cell debris in central cavities of the rosette, ultrastructural features of radial glial cells, including cell coupling zonula adherens and tight junctions, deposits of glycogen storage granules and cilia. Additionally we also observed a network of smaller,

multipolar cells among the rosettes that contained smaller glycogen deposits and lipid droplets. Finally we observed mitotic cells both in the vicinity of the central cavities and further away from the center of the rosettes. In summary our microscopic results clearly demonstrated that neural rosettes were observed in both induction methods independent of cell line differences.

3.2.1 Generation of PAX6+/NESTIN+ neural progenitor cells

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, FACS and q-PCR. FACS analysis showed that the proportion of NESTIN and PAX6 double positive cells in 2D was 83% (±1.73 SEM) and in 3D was 93.34% (±1.55 SEM) ($p<0.05$). Thereby indicating 3D was superior to 2D. On the other hand mRNA level indicated an in consistence expression levels in both the genes *NESTIN* and *PAX6* with both induction methods. Taken together, the overall result based on quantitative FACS results indicates that the proportion of $PAX6^{+}$ and $NESTIN^{+}$ NPCs was significantly higher on using the 3D neural induction method.

3.2.2 Generation of neural progenitor cells and glial progenitor cells

Recent studies have suggested that the transcription factor SOX9 is highly enriched in astrocytes. To identify a nuclear marker of astrocytic phenotype, we quantified the SOX1 population separately and SOX9 population separately using FACS. The analysis have indicated a significantly higher expression of SOX1 in 2D cultures (86.1% (±2.02 SEM)) compared to 3D cultures (76.4% (±1.8 SEM)) indicating that 2D neuronal induction are more efficient in generating neuronal population. Subsequently when analysed the amount of glial subpopulation in generated NPCs we observed a modest increase in SOX9 subpopulation in 3D cultures (12.3 %(±0.961 SEM)) while a decent in 2D cultures (8.51% (±0.838 SEM). Thus clearly indicating that 3D neuronal induction can constitute to astrocytes generation when appropriate growth factors are involved for astroglial generation. This is in corresponds to (Shaltouki et al., 2013) where their study is based on 3D spheres for astrocytes generation. Based on quantitative FACS data we can conclude that, the 2D neural induction method generated more SOX1 positive neural progenitor cells in all of our examined cell lines.

To conclude we can say that neuronal precursor cells (NPCs) were successfully generated with both induction methods. However the 2D neural induction method generates more SOX1 positive neural progenitor cells while the 3D neural induction method generates double positive NESTIN and PAX6 cells.

3.2.3 Terminal differentiation capacity of the NPCs

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 and 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. 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.

3.2.4 NPCs fate determinant

We then examined the expression of the cortical layer markers *CUX1* (*cut-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). Our results showed 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.

Subsequently, we also evaluated the expression of 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]. 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 express 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.

3.2.5 Neurite Outgrowth Capacity

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 neurons (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 and a remarkable and significant difference was observed within the differentiated neurites, suggesting the 3D method is more superior for neurite elongation.

3.2.6 Electrophysically Activity

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), 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). 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.

3.3. 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.

3.3.1 Rapid generation of mouse 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.

(i) 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. (ii) 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. (iii) 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).

3.3.2 Human astrocytes generation from hiPSCs

From our human studies, we generated about >85% SEM±16.12 of GFAP and AQP4 double positive cells within 5 weeks. Additionally, we observed a steady increase in terminally differentiated neurons after 3 weeks of culture.

As a novel approach we were able to generate *in vitro* astrocytes on their phenotypic features (GFAP and AQP4) both in mouse and human pluripotent cells.

4. NEW SCIENTIFIC RESULTS

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.

5. DISCUSSION AND SUGGESTION

5.1 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.

5.2 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.

5.3 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.

5.4 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.

6. PUBLICATION

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.
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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.
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- **Chandrasekaran A** Patient specific induced pluripotent stem cells (iPSCs) and their neuronal differentiation Utrecht University, Netherlands. 13th June 2016.

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