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CELLULAR MODELS OF HUMAN BRAIN DISORDERS FROM SKIN TO BRAIN

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Cellular models of human brain disorders
From skin to brain

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For my mom, for her love
For Shahin, for everything

ABSTRACT

Studying human brain development and disorders is very challenging. In the absence of comparable model organisms, human-related models, and limitations to obtain live cells from the human brain, induced Pluripotent Stem Cell (iPSC) technology, in particular, provided a unique tool to study the disease mechanisms and investigate potential treatments. The main goal of this thesis was to study neurological disorders and explore novel mechanisms underlying the diseases.

We have generated and characterised patient and healthy control iPSCs using *Sendai virus* as a safe non-integrating method to keep the host genome intact. We have then shown an example of a standardised culture condition by using recombinant spider silk coating for iPSCs and Embryonic Stem Cells (ESCs) cultivation in 2D and 3D formats. Healthy Pluripotent Stem Cell lines cultured on recombinant spider silk displayed the typical stem cell morphology with the expression of pluripotent stem cell markers. Considering the xeno-free culture coating and compatibility with the host immune system, the spider silk, can provide an optimal routine culture system for pluripotent stem cells and future iPSC based therapies.

Patient and healthy iPSCs were neurally induced to generate intermediate, expandable Neuroepithelial Stem Cell (NESC) lines. Morphologically, the derived NESCs displayed rosette structures in culture and expressed key neural stem cell markers. Also, the transcriptomic profile of derived lines displayed similarity that proposes the homogeneity of our NESC population despite patient genomic background variation. We have used a healthy control NESC line to model Alzheimer's Disease (AD) in a dish by exogenous application of amyloid beta oligomers in differentiating culture. Interestingly, AD-related phenotype, dis-localisation of phosphorylated P21-activated kinases (pPAK) protein, was recapitulated only in 3D culture.

We further attempted to identify mechanisms underlying Type 1 Lissencephaly from patients carrying Doublecortin (DCX) mutations. Differentiating patients' cells with dis-regulation of DCX expression exhibited a migrational defect, aberrant neurite outgrowth, and fewer dendrite bundles. In addition, we have dissected a proliferation phenotype of DCX mutant cells upon differentiation. Data suggests an indispensable role of DCX expression at an early stage of neural development which allows proper differentiation and migration.

Here we have shown that it is possible to make a robust cellular model to study human brain disorders using patient-specific cells. Identification and verification of disease phenotypes and exploring the underlying mechanisms could provide valuable insights into these complex disorders. These insights may offer novel approaches to therapeutic applications taking scientists one step closer to treating the patients. This study underlines the importance of cellular-based models, 2D and 3D, that can be used to study typical development as well as disease mechanisms.

LIST OF SCIENTIFIC PAPERS

I. Wu S, Johansson J, Damdimopoulou P, **Shahsavani M**, Falk A, Hovatta O, Rising A.
Spider silk for xeno-free long-term self-renewal and differentiation of human pluripotent stem cells.
Biomaterials, 2014 Oct;35(30):8496-50.

II. Zhang D, Pekkanen-Mattila M, **Shahsavani M**, Falk A, Teixeira AI, Herland A.
A 3D Alzheimer's disease culture model and the induction of P21-activated kinase mediated sensing in iPSC derived neurons.
Biomaterials, 2014 Feb; 35(5):1420-8.

III. **M Shahsavani**, RJ Pronk, R Falk, M Lam, M Moslem, SB Linker, J Salma, K Day, J Schuster, B-M Anderlid, N Dahl, FH Gage and A Falk.
An *in vitro* model of lissencephaly: expanding the role of DCX during neurogenesis.
Molecular Psychiatry, 2017 Sep 19. doi:10.1038/mp.2017.175

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LIST OF ABBREVIATIONS

Aβ	Amyloid beta peptide
AD	Alzheimer's Disease
aGSK-3b	active glycogen synthase kinase-3b
ALS	Amyotrophic Lateral Sclerosis
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
ASD	Autism Spectrum Disorder
bFGF	basic Fibroblasts Growth Factor
BMP9	Bone Morphogenic Protein
CDH2	Cadherin-2
CDK5	Cyclin-dependent Kinase 5
CHL1	Cell adhesion molecule L1-like
CRISPR/CAS9	Clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9
DACH1	Dachshund homolog 1
DCX	Doublecortin
DS	Down Syndrome
EBs	Embryoid Bodies
EGF	Epidermal Growth Factor
EHS	Engelbreth- Holm-Swarm
EBV	*Epstein–Barr virus*
FGF	Fibroblasts Growth Factor
FXS	Fragile X Syndrome
GO	Gene Ontology
HDAC	Histone Deacetylase
hESCs	human Embryonic Stem Cells
HFF	Human Foreskin Fibroblast
hiPSCs	human induced Pluripotent Stem Cells
Ile	Isoleucine
IRES2	Internal Ribosomal Entry Site 2
JNK	c-Jun N-terminal Kinases
LIF	Leukemia Inhibitory Factore
LIN28	Lin-28 homolog A
MET	Mesenchymal to Epithelial Transition
mDAn	midbrain Dopaminergic neurons
MMRN1	Multimerin-1
Myc	Myelocytomatosis oncogene
NESCs	Neuroepithelial Stem Cells
NGF	Neurve Growth Factor
NSC	Neural Stem Cell

NTNG1	Netrin G1
Oct-4	Octamer-binding transcription factor 4
PKA	Protein Kinase A
PLZF	Promyelocytic Leukemia Zinc Finger
PAK	P21-Activated Kinases
pPAK	phosphorylated P21-Activated Kinases
PP1	Protein Phpsphatase 1
PPP1R9A	Protein Phosphatase 1 Regulatory Subunit 9A (Neurabin1)
PS1	Presenilin 1
PS2	Presenilin 2
4RepCT	A recombinant miniature spidroin consisting of four poly-Ala/Gly-rich tandem repeats and a nonrepetitive C-terminal domain
RELN	Reelin
RTT	Rett Syndrome
SBH	Subcortical Band Heterotopia
Ser/Pro	Serine/Proline
SeV	*Sendai virus*
SEZ	Subependymal Zone
Shh	Sonic hedgehog
SMAD	homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the Caenorhabditis elegans protein Sma
SOX2	SRY (sex determining region Y)-box 2
SSEA	Stage-Specific Embryonic Antigen
SV40LT	Simian Vacuolating virus 40 Large T antigen
SZ	Schizophrenia
SVZ	Subventricular Zone
TCF7L2	Transcription factor 7-like 2 ((T-cell specific, HMG-box)
TGF-β	transforming growth factor-β
TH	Tyrosine Hydroxylase
TRA1	Tumor Rejection Antigen 1
TS	Timothy Syndrome
TUBA1A	Tubulin Alpha 1A
WHO	World Health Organisation
Val	Valine
VPA	Valproic Acid
ZO-1	Zonula Occludens-1

1 THE STUDY OF HUMAN BRAIN DEVELOPMENT AND DISORDERS; CHALLENGES AHEAD

Studying the human brain has always been complicated. Besides ethical issues, it is not feasible to directly study the human brain due to its location and inaccessible anatomy. Understanding mechanisms of human brain disorders, specifically those with a developmental origin, have been very challenging, as taking live cells from patients is very rare. Moreover, postmortem samples are not commonly available and give limited information. Although animal model studies reveal precious information, it is not easy to translate animal data to human, due to species differences, the complexity of the human brain, and diseases related solely to the human brain. There is a need for cellular models that mimic the human brain to study and understand the mechanisms underlying the disease and reduce the hindrances in development of new treatments.

After successes in culturing embryonic stem cell (ESC) lines from inner cell mass of mouse blastocyst by Matthew Kaufman and Martin Evan [1] and Gail Martin [2] in 1981 and later on of human by Thomson and colleagues [3], ESCs have been used as an amenable tool to study the developmental process as well as disease mechanisms with the perspective of applying them as a source of transplantation and therapeutic purposes. *In vitro* culture of pluripotent ESCs revealed their limitless self-renewal capacity and differentiation potential into the three germ layers; ectoderm, endoderm, and mesoderm. Some studies have used ESCs to investigate the normal development and disease mechanisms such as Fragile X Syndrome and Lesch-Nyhan disease [4], [5]. Due to limitations in using ESCs, such as ethical implications, hESCs accessibility, and availability, there was an unmet demand for an *in vitro*, unlimited, available, and autologous source of cells that could feasibly be generated and accessible in the lab.

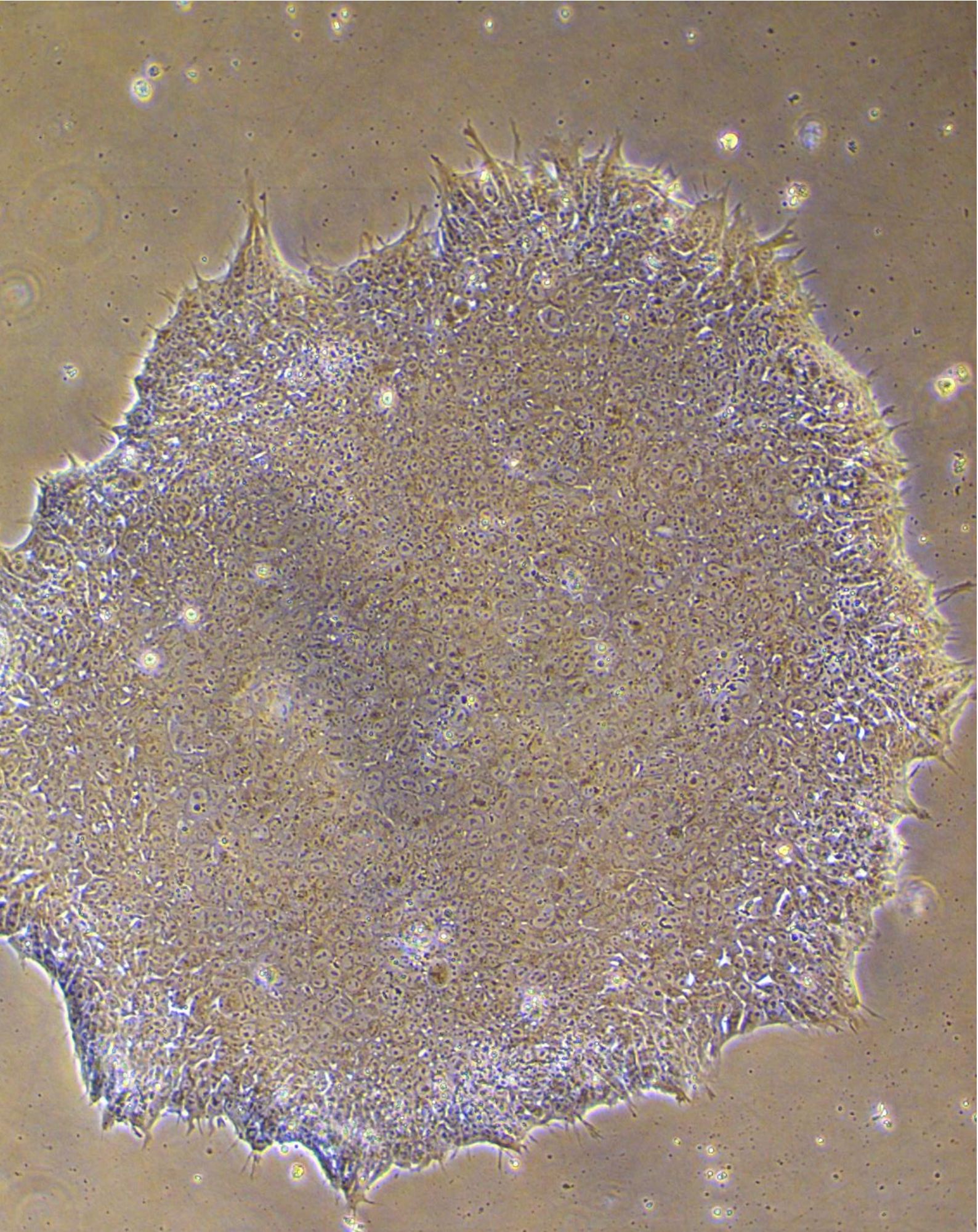
Shinya Yamanaka made this possible and was deservedly awarded the Nobel Prize in Physiology and Medicine 2012 jointly with Professor John Gurdon. In 2006 Yamanaka and his colleague introduced a combination of essential pluripotent genes into fibroblasts, first in mouse [6] and later in human fibroblasts [7]. He was able to turn back the developmental clock from fully differentiated cells to the embryonic stage. He started with ectopic co-expression of 24 pluripotency-associated candidate genes that eventually narrowed down to just four factors;

Oct4 (Pou5f1), Sox2, Klf4, and c-Myc. Reprogrammed cells were termed induced pluripotent stem cells (iPSCs) by Yamanaka. Thomson laboratory also reported the successful human fibroblast reprogramming by using 4 transcription factors Oct4, Sox2, Nanog, and Lin28 in the same year [8].

iPSCs resemble ESCs with respect to morphology, gene expression profile, and expression of cell surface markers. iPSCs are pluripotent; they have unlimited self-renewal capacity and the ability to differentiate into the three germ layers; ectoderm, endoderm, and mesoderm. Reprogramming from a wide range of somatic cells from human enables scientists to generate induced pluripotent stem cells in the lab. iPSCs have been shown to be equivalent to human embryonic stem cells which makes them a perfect *in vitro* model to study human development in a dish.

By reprogramming somatic cells, typically fibroblasts, we have been able to derive patient-specific stem cells. With access to patient-specific iPSCs, we can further differentiate them towards specific cell types relevant to the disease. Studies evaluating impaired neurogenesis and migration, neuronal connectivity, synaptic plasticity and functional electrophysiology would be valuable in order to understand disease-related phenotypes [9], [10]. Besides modelling disease, iPSCs can be used in drug discovery to screen possible drugs or develop where they cannot be tested in humans [9] and even the possibility to develop tailored treatments. Moreover, they have therapeutic potential and are considered good candidates for autologous cell transplantation [11]. A future perspective would be to apply gene targeting for known mutations, to create iPSCs with corrected DNA and differentiate them to the affected cell type, which would finally allow healthy cells to be transplanted back to the patient [12], [13].

In recent years, several neurological disorders have been modeled using iPSCs such as Autism Spectrum Disorders (ASDs) [14], [15], Timothy Syndrome (TS) [16], Fragile X Syndrome (FXS) [17], [18], Rett Syndrome (RTT) [19], Down Syndrome (DS) [20], [21], Alzheimer's Disease (AD) [22], [23], Amyotrophic Lateral Sclerosis (ALS) [24], [25], Schizophrenia (SZ) [26], and the number is growing, showing how powerful the technique is to recapitulate and discover the disease-related phenotypes and use the knowledge to hopefully help patients with such complex diseases.



A typical induced pluripotent stem cell (iPSC) colony on laminin-521 coating

2 INDUCED PLURIPOTENT STEM CELLS

Reprogramming occurs through key sequential events at cellular and molecular levels reviewed in [27]. During the initiation phase, somatic cell signature is lost via downregulation of somatic differentiation genes, leading to morphological changes of the cells known as MET (mesenchymal to epithelial transition). At the same time, upregulation of pluripotency markers such as SSEA1 and alkaline phosphatase (AP) appear. Transduced cells gain proliferation capacity likened to ESC's, as well as inhibition of apoptosis and senescence, due to the vast transcriptional changes induced by the reprogramming factors. It has been shown that transcription factors Oct4, Sox2, and Klf4 act as ''pioneer factors'' and initially bind to inactive DNA regions with the *c*-Myc cooperation to turn on pluripotency. Moreover, c-Myc binds to open chromatin regions suppressing fibroblast-specific genes [28], [29]. Next comes maturation phase with the gradually increased expression of some pluripotency markers such as NANOG and endogenous OCT4. During the final phase, cells that passed the maturation phase acquire a pluripotency signature by expressing a second subset of pluripotency genes such as SOX2, resulting in the production of bona fide iPSCs that are independent of ectopic expression of reprogramming factors.

The epigenetic reprogramming is as crucial as the gene reset during the reprogramming process. It is believed that epigenetic reprogramming begins with global demethylation of different genes before the actual gene silencing and activation occur. The global gene demethylation is prior to the functioning of the reprogramming factors. This is mainly mediated by ESC-specific microRNAs such as micR-302 family [30], [31].

iPSCs have unlimited self-renewal capacity, express pluripotent stem cell markers including cell surface markers TRA1-60 and TRA1-81 and pluripotent cell nuclei markers OCT4 and NANOG. The differentiation potential of iPSC lines is assessed *in vitro* by embryoid bodies (EB) formation and *in vivo* by teratoma formation. A potential replacement to teratoma assay which has been recently introduced is Pluritest; a bioinformatics and noninvasive method. PluriTest is a panel of around 450 genome-wide transcriptional profiles of which 223 are hESC lines and 41 are hiPSC lines, and the rest are from various differentiated cell types and tissues from developing and adult human all from a variety of laboratories [32].

The first generation of iPSC lines did not display fully reprogrammed cells equivalent to ESCs. The DNA methylation status and gene expression patterns of derived lines were different to ESC lines; the promoter of key reprogramming mediators such as Oct4 displayed incomplete demethylation [6]. After uncovering these imperfections, Yamanaka and other laboratories attempted to redesign the experiment and improve the technique. They successfully demonstrated the high similarity of iPSCs and ESCs at the epigenetic state and transcriptional profile [33], [34]. It has been shown that besides morphology and functional similarity between iPSCs and ESCs, the epigenetic state and transcriptional profile are both highly similar [34]. It is believed that reprogramming itself is not a primary issue that causes differences in derived iPSC lines, but rather other technical factors including transduction method and culture conditions. Due to the events during reprogramming that cannot be controlled, it is important to select good iPSC clones to obtain a proper cellular model [35].

Here I discuss two important fundamental factors that directly affect reprogramming leading to a complete and perfect iPSC generation; the gene delivery method and culture condition.

2.1 INTEGRATION-FREE MEDIATED REPROGRAMMING

The induction method greatly affects the efficiency of reprogramming and the quality of derived iPSCs. Yamanaka used *retrovirus* to introduce pluripotency genes into fibroblasts. Since then, reprogramming has been performed with multiple methods such as *lentivirus*, *adenovirus*, plasmids, transposons, *Sendai virus*, protein delivery, microRNA, and chemical compounds [12]. *Retrovirus* and *lentivirus,* initially the most commonly used, are enveloped single-stranded positive-sense RNA viruses. Upon entering the host cell, their RNA is reverse-transcribed into DNA, which is then integrated into the host genome, followed by the regular transcription and translational processes to express the genes carried by the virus. The genomic integration can cause insertional mutagenesis, disrupt the target cells, and alter gene expression patterns, which can compromise drug screens, disease pathway analyses, and also increase the risk of tumorigenesis. Additionally, these vectors have very low efficiency ~0.001-1%; the number of resulting iPSC colonies per the number of infected cells seeded gives us the reprogramming efficiency.

In contrast, non-integrating vectors such as *adenovirus* and *Sendai virus*, direct protein delivery, episomal vectors, and synthetically modified mRNA do not integrate the host genome, which can decrease the risks associated with the DNA-based integrating methods. *Sendai virus* transduction, as a DNA free method, has very high efficiency, ~0.1-1%, that is 100-fold compared to standard methods and does not require multiple rounds of transfection which is required for some vectors [36], [37]. *Sendai virus* is an enveloped virus with a negative-sense single-stranded RNA. After infection, the virus goes through genome replication and protein synthesis in the host cytoplasm, and then daughter virus particles are assembled and released keeping the genome intact [36], [38]. *Sendai virus* vector is considered as a safe method for clinical studies of gene therapy for cystic fibrosis [39], [40] as well as gene vaccine delivery [41].

microRNAs mediated reprogramming has become more important recently. miRNAs are able to facilitate the reprogramming through the induction of reprogramming factors such as miR-302 [30] or even induce the reprogramming on their own expression [42], [43]. It has been shown that overexpression of miR302/367 cluster is enough to reprogram mouse and human fibroblasts with high speed and efficiency without using conventional reprogramming factors.

Modified synthetic mRNAs encoding the reprogramming factors Oct4 (Pou5f1), Sox2, Klf4, and c-Myc have been shown to successfully generate iPSCs with high efficiency [44]. mRNA mediated reprogramming was performed in combination with B18R interferon inhibitor to avoid the innate antiviral responses. This suggests the faithful application of mRNA reprogramming in regenerative medicine and therapeutic stem cell clinical application.

Another non-integrating method is using episomal vectors. Episomal vectors are considered as a safe method and suitable for clinical grade applications. Yamanaka reprogramming factors plus Nanog, Lin28, SV40 LT, as well as IRES2 a co-expression factor, have been introduced to vectors with the *Epstein–Barr virus* (*EBV*) origin [45]. This method was limited to using different cell types and low efficiency. In another study using p53 suppression and nontransforming L-Myc, the efficiency was significantly increased, and iPSCs were successfully derived from various donors [46]. A more recent study demonstrated higher efficiency in the episomal reprogramming of Oct4, Sox2, and Klf4 with no oncogene ectopic expression of c-Myc and Lin28 [47].

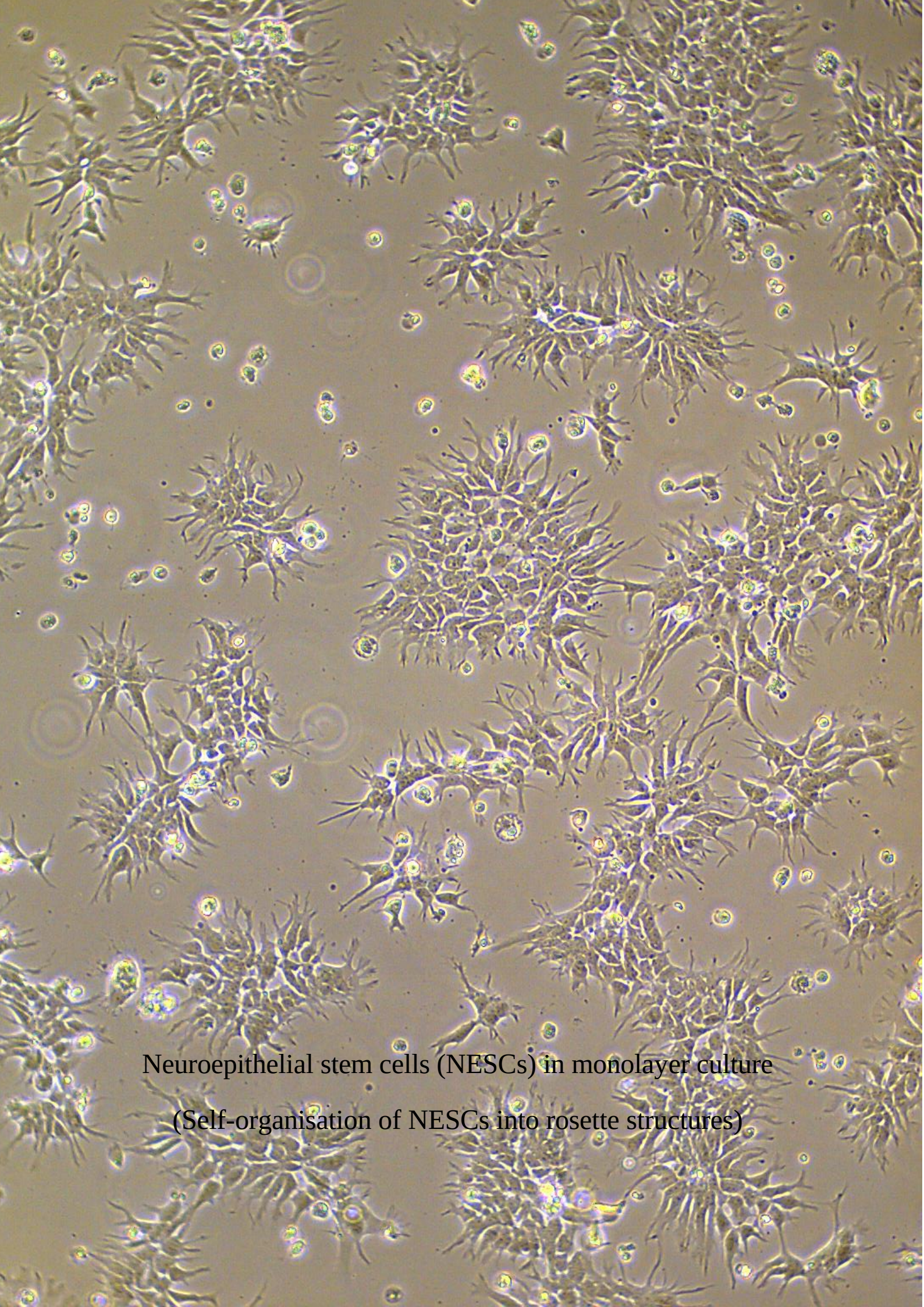
Direct protein delivery has also been reported as a safe method for iPSCs derivation [48]. Oct4, Sox2, Klf4, and c-Myc recombinant proteins were fused with a cell-penetrating peptide (CPP) and used as reprogramming factors to treat somatic cells. Protein-induced iPSC method was very slow and lacked high efficiency; however, this method excluded the risk of using any DNA and vectors. Application of small molecules has also been considered as a promoting factor in some reprogramming methods. In a protein-induced method, reprogramming proteins generated iPSCs in the presence of valproic acid (VPA), a small-molecule HDAC inhibitor [49], [50]. It has been shown that reprogramming is doable without the oncogenes Klf4 and c-Myc if VPA is applied during the *retrovirus* reprogramming process [51].

It is now routine in many laboratories to use non integrating methods such as *Sendai virus* or plasmids for reprogramming. Although all integration-free methods are prominent for iPSC application in the clinic, it should be noted that optimisation is required before being applied in clinical trials.

2.2 XENO-FREE FEEDER-FREE IPSC CULTURE COATING

Cultivation of induced pluripotent stem cells is as crucial as reprogramming. Correct cultivation supports full reprogramming and maintains the stem cell pluripotency and homogeneity at a high level and prevents differentiation. Culture conditions can change the gene expression profile and influence the stem cell characteristics and further downstream applications. To provide an efficient cellular model, it is essential to optimise and standardise the iPSCs culture condition. One fundamental factor is an effective coating system as stem cells widely interact with each other and the extracellular matrix.

Initially, human pluripotent stem cells were cultured on feeder layers obtained from inactivated mouse embryonic fibroblasts (MEFs) [3], [7]. Later, inactivated human foreskin fibroblasts (HFFs) have also been used as feeder layers for expanding pluripotent stem cell lines accordingly [52]. Using the mouse and human feeder layers both raise the concern of contamination as well as the presence of non-human and unmatched immunogenic epitopes which impede the clinical applications of pluripotent stem cells. Therefore, feeder-free cultures were considered as an alternative method. The first used substrate was Matrigel; a basement membrane extracted from mouse Engelbreth-Holm-Swarm (EHS) tumour that consists of certain macromolecules; mainly type IV collagen, laminin, the heparan sulfate proteoglycan, and entactin [53], [54]. Matrigel supports stem cell growth while cells maintain stem cell characteristics similar to feeder layer including self-renewal maintenance, pluripotent gene expression, and teratoma formation ability. Later on, several extracellular matrix proteins such as Vitronectin, laminin-521, E-cadherin, and Fibronectin have been used as a feeder-free culture. Besides the sizeable batch-to-batch matrigel variability, these substrates are of xenogeneic origin [55]; therefore a suitable and xeno-free culture is still required to be able to use the pluripotent stem cells for pharmaceutical and regenerative medicine applications. Recently the focus has been on developing synthetic based substrates like peptide and polymer-based substrates, and hydrogels. Furthermore, a host adaptable system as well as a three dimensional (3D) scaffold with the aim of imitating the *in vivo* environment, have been considered for iPSC applications.



Neuroepithelial stem cells (NESCs) in monolayer culture

(Self-organisation of NESCs into rosette structures)

3 HOW TO MIMIC THE *IN VIVO* SITUATION TO CAPTURE DISEASE-RELATED PHENOTYPES

To establish a good cellular model and recapitulate the disease-related phenotypes, it is important to understand the disease hallmarks and its pathophysiology in order to focus on generating relevant cells that are associated with the disease. Studying the endpoint samples of diseases are not very helpful because the disease has already been developed. Moreover, this does not help us to understand the development, onset, and the progression of the disease which might be more crucial. It is believed that causal genes for neurodevelopmental disorders take effect during the early embryonic stage of fetal development. Regarding neurodegenerative diseases, even if the onset of the diseases appears in adulthood, the phenotypic differences can be present in early neural development. In addition, transcriptome profile and functionality assays revealed that the *in vitro* derived iPSC neurons are immature neurons and equivalent to fetal neurons. This unique similarity allows us to use them to study neurodegenerative disorders as well as neurodevelopmental disorders during neural development [56], [57]. Therefore, I believe that neural progenitor cells, which represent an early stage in the neural tube during development, are a good candidate for *in vitro* studies of neurological disorders.

In 1982, Brent A. Reynolds and Samuel Weiss for the first time isolated neural stem cells from the striatum of the adult mouse brain and cultured them as monolayer and spheres. Isolated cells were Nestin positive and proliferated in the presence of EGF and were capable of differentiation to CNS neurons and astrocytes [58]. Later on, it was shown that isolated progenitors from adult mouse striatal tissue were multipotent and characteristic of stem cells. Neural stem cells were cultivated in the presence of bFGF [59]. The neural progenitors/stem cells have been isolated from many regions of the human brain such as SVZ [60], SEZ and hippocampus [61], and cortex [62]. By isolating neural stem cells from various parts of the brain, attempts were focused on *in vitro* neural induction from ESCs and later iPSCs. Initially, induction protocols were performed by spontaneously differentiating EBs or using stromal feeder [63], [64]. However, none of these protocols was defined.

In vitro derivation of the neural stem cells is contingent with the understanding of *in vivo* processes. The nervous system initially develops from the neural plate from the embryonic ectoderm in the 3rd week after fertilisation. The neural plate is induced by a number of coinciding signals including BMPs, FGF, Shh, and Wnts which all interact synergically in a spatial and temporal manner leading to neural induction. BMP signalling is one of the key regulators of ectoderm transformation that controls the cell fate decision, proliferation, and patterning. BMP proteins, belonging to transforming growth factor-β (TGF-β) protein family, bind to the BMP receptors and trigger the signal via activation of Smad proteins. This signal results in translocation of activated Smad complexes to the nucleus to regulate the expression of the target gene with the help of other nuclear cofactors. The BMP antagonists, such as Noggin, I-Smads, and Chordin, can block the BMP signal allowing the ectoderm to procure the neural fate. Active suppression of BMP signalling is known to be a general mechanism for neural induction [65]–[67].

Based on *in vivo* neural induction, Lorenz Studer's laboratory reported a dual-SMAD-inhibition protocol as an efficient *in vitro* derivation of neural stem cells from hESCs and hiPSCs [68]. Simultaneous application of Noggin and SB431542, two inhibitors of SMAD signalling, increases the neural induction efficiency and yields a higher number of neural stem cells in comparison to conventional EBs formation or stromal feeder-mediated induction methods.

Recent studies on hESCs and hiPSCs have shown the derivation of stable neural progenitor cell lines as an expandable intermediate cell population to study early stages of development [69], [70]. The capture of these progenitors is quick, efficient, and reproducible, while the cells maintain neural progenitor/stem cell characteristics. The rosette-forming cells isolated from neurally induced pluripotent stem cells are propagated in serum-free medium supplemented with bFGF and EGF while retaining a high self-renewal capacity, as well as high neurogenic potential upon differentiation. These cells, named long-term self-renewing neuroepithelial-like stem cells: lt-NES cells hereafter called NESCs. NESCs are expandable for 100 or more passages without losing their neurogenic potential. NESCs express neural precursor markers Nestin and SOX2, as well as neural progenitor cell markers SOX1 and PAX6. Derived NESCs

are also positive for rosette marker transcription factors PLZF and DACH1, as well as a polarity marker of neuroepithelial cells ZO-1 which is expressed apically and indicates the polarised organisation of NESCs in culture [71]. Different types of neuronal subtypes can be acquired from NESCs by either bFGF and EGF removal or more specifically, applying extrinsic factors inspired from *in vivo* development; forebrain cholinergic neurons [72] and midbrain dopaminergic neurons (mDAn) [73]. It has been shown that NESCs are developmentally similar to neuroepithelial stem cells captured from the human fetal brain [74]. NESCs have been considered as a worthwhile tool for *in vitro* disease modelling [75]–[77] as well as the potential for use in high-throughput screening applications (HTS) [78]. A very recent study also showed the potential of NESCs to make functional astrocytes ‘‘NES-Astro’’ in a defined condition [79].

In the next 2 sections, I will describe examples of neurological brain disorders where cellular models have been used to uncover disease mechanisms; Alzheimer’s Disease as a neurodegenerative disorder and Type 1 Lissencephaly as a neurodevelopmental disorder.

4 ALZHEIMER'S DISEASE

Alzheimer's disease is the most common form of dementia that affects millions of people around the world. According to the World Health Organization (WHO), approximately 35 million people around the world are suffering from AD. The rate of disease is rising quickly while there is no promising prevention, neither effective treatment. The disease gives a progressive neurodegeneration of the brain, where the forebrain cholinergic neurons are affected early in the disease process and results for example in impaired memory. The neurodegeneration can correlate with the two major neuropathological features of AD, the presence of large numbers of extracellular senile (neuritic) plaques and neurofibrillary tangles. Amyloid plaques comprise of the amyloid beta peptide (Aβ) aggregates, surrounded by dystrophic neurites and glial cells. Neurofibrillary tangles are abnormal intraneuronal proteins organised in bundles of paired helical filaments, composed of hyperphosphorylated forms of the microtubule-associated protein Tau [80], [81]. The disease is multifactorial, and it is estimated more than one hundred genes are involved. AD is categorised into two types, late onset and early onset. The late onset of AD is the most common form and occurs after age 65. There is no defined mutation or any single cause leading to the disease; however, ApoE polymorphism (ε4) has been recognised as a significant risk factor [82]. This type is called sporadic AD. In contrast, familial AD is rare, runs in families and ordinarily has early-onset, usually under age 60 [83]. It has been established that mutations in genes responsible for Aβ peptide production (amyloid precursor protein (APP), and two components of the γ-secretase enzyme; presenilin1 (PS1) and presenilin2 (PS2)) lead to familial AD. Aβ peptides consist of 36 to 43 amino acids that are typically produced from APP by sequential cleavage of the enzymes β-secretase and γ-secretase. One of the known mutations in APP is a point mutation in exon 17 on chromosome 21 that replaces a Val → Ile, known as London mutation (Val717Ile) [84]. Aβ monomer is the normal form of the protein. However, in the disease process, these peptides can form oligomers and insoluble aggregates that are one of the pathological hallmarks of the disease [83], [85].

Despite the fact that AD is ordinarily human-related and knowing animal models are not capable to fully recapitulate human brain diseases, several cellular and animal models have been used to uncover AD mechanisms and discover potential treatments [86]. For example, a transgenic mouse model with APP mutation (V717F) showed the presence of amyloid plaques extracellularly in the mouse brain, but not neurofibrillary tangles. The Aβ accumulation associated with distorted neurites, synaptic loss, as well as dendrite reduction in the AD mouse hippocampus [87]. Another transgenic mouse model reported that Tau reduction did not influence the Aβ associated pathology, but instead prevented the cognitive deficits in AD mice. This result suggests that decreasing endogenous Tau protein could be a potential treatment for the disease [88].

Recent studies have shown that it is possible to model the disease *in vitro* and capture different aspects of the disease despite the fact that *in vivo* symptoms do not appear until later in life. The first iPSC-based model of AD reported increased Aβ42 secretion in neurons derived from familial AD patients carrying PS1 or PS2 mutations. This model demonstrated the recapitulation of the disease-related pathology in a dish. The elevated Aβ 42/40 ratio was rescued with γ-secretase inhibitor treatment [22]. In another study, iPSC-derived neurons from two familial AD patients with APP duplication and two sporadic AD patients showed an increase in Aβ40 secretion, phosphorylated Tau, and aGSK-3b. Treatment with β-secretase inhibitor could rescue the elevated phosphorylated Tau protein and aGSK-3b, but not Aβ40 [23].

More specifically, cholinergic iPSC derived neurons from sporadic AD patients with ApoE3/E4 genotypes, represented AD pathology including increased Aβ42/Aβ40 ratio and response to γ-secretase inhibitor treatment [89]. Derived neurons were more susceptible to increased glutamate toxicity and cell death. Forebrain neurons derived from familial AD patients with the London mutation showed significantly elevated Aβ, Aβ42/40 ratio, and Tau protein levels compared to control neurons [90]. The total and phosphorylated Tau protein levels could be rescued via Aβ depletion. Specific monoclonal and polyclonal anti-Aβ antibodies treatment sequestered the Aβs and decreased the total Tau protein level only at the early stage of differentiation.

To recapitulate more aspects of this complex disease and uncover the mechanisms, recent focus has been on developing more enhanced *in vivo* related models such as 3D neurosphere culture and brain organoid culture systems. Tau neurofibrillary tangles, one of the AD hallmarks, was recapitulated for the first time in the 3D culture of a genetically engineered human NSC line overexpressing several familial AD-associated genes [91], [92]. A recent study reported the presence of Aβ and Tau protein aggregates in neurosphere culture from engineered human NSC and iPSC lines with familial AD [93].

5 LISSENCEPHALY

Lissencephaly is a neuronal migration disorder that is considered to have a neurodevelopmental cause. In the developing brain between 5 and 22 weeks of gestation, immature neurons derived from neural stem cells in the ventricular zone migrate over hundreds of cell body distance from their sites of origin toward the cortical plate in an inside-out manner to build the six cortex layers. Mutations in several genes such as LIS1 (PAFAH1B1), DCX, and TUBA1A are involved in neuronal migration leading to lissencephaly [94]. Lissencephaly (smooth brain) explains a range of brain malformations characterised by a smooth cerebral surface, resulting in developmental delay, many neurological disabilities, and seizures. Mutated cells fail to migrate correctly from the ventricular zone to the cortex during brain development, resulting in the development of a poorly organised cortex without regular folds and ridges. Based on disease pathogenesis Lissencephaly is classified into two classes; the classical Lissencephaly (Type 1 Lissencephaly) and subcortical band heterotopia (SBH). Patients with Type I Lissencephaly carry a mutation in one of the migratory related genes resulting in the development of a thickened cortex with absent (agyria) or reduced folding. In contrast, SBH patients develop a grey heterotopic band beneath the cortex located in the central white matter (Figure 1). SBH often occurs in females and presents with less severe malformation compared to the classical Lissencephaly affected patients [95].

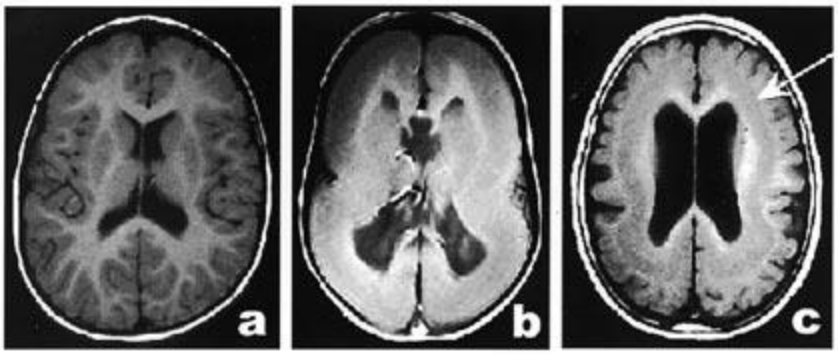


Figure 1 T1-weighted magnetic resonance images of a healthy brain compared with two Lissencephaly patient brains. (a) A healthy brain; (b) Classical Lissencephaly with a mutation in DCX gene (c) SBH in a female child with a mutation in DCX gene, the arrow indicates the subcortical band. Picture adapted from reference [96].

LIS1 is the first discovered gene that causes Lissencephaly associated with Miller-Dieker Lissencephaly syndrome [97]. The gene is located on chromosome 17 and encodes the platelet-activating factor acetylhydrolase (PAFAH1B1). LIS1 plays roles in a variety of biological processes such as functioning as a regulatory protein activator factor, involvement in neuronal migration, and axon growth. LIS1 interacts with DCX and the microtubule motor cytoplasmic dynein to regulate microtubule dynamics, in addition to cell division [98], [99].

TUBA1A gene located on chromosome 12 belongs to the tubulin superfamily. The gene corresponds to tubulin alpha 1a protein which is abundant in the developing brain and is a main component of microtubules. TUBA1A is essential for proper microtubules function which explains its involvement in cell motility and neuronal migration [100].

Doublecortin is the major causative gene of X-linked Lissencephaly. Males with hemizygous DCX mutations result in the classical form of Lissencephaly (Type 1 Lissencephaly) which shows a severe phenotype (Figure 1b). Females with heterozygous DCX mutations represent SBH Lissencephaly with a genetic mosaicism. Due to random X-inactivation, two populations of migrating neurons are produced. One population expresses the normal DCX allele and migrates correctly to establish the outer cortex. The other, with the mutated DCX allele, fails to adequately migrate resulting in the formation of the heterotopic band of neurons (Figure 1c) [101]. The gene encodes the brain-specific microtubule-associated protein consisting of 360 amino acids with a 40 kDa predicted protein [102], [103]. DCX is expressed in neural progenitors and immature neurons and is abundant in the cell soma as well as tips of neurites. DCX acts in the migration of neural stem cells from the ventricular zone to their final anatomical destinations to build up the six layers of the cortex. DCX expression is regulated during brain development [104] and so far has been used widely as a neurogenesis marker. The expression is sustained in migratory neuroblasts as well as adult neurogenesis regions, the dentate gyrus and the lateral ventricle wall/olfactory bulb axis [105].

DCX function and involvement in every step of neuronal migration is currently poorly understood. DCX localisation was demonstrated for the first time by Gleeson in 1999 suggesting a distinct expression in the periphery of the cell soma surrounding the nucleus and

expanding to the proximal neurite [104]. DCX co-localises with microtubule compounds such as actin suggesting its association with microtubule cytoskeleton through stabilising the microtubules. Most likely, DCX does not support microtubule growth, but instead prevents de-polymerisation and thus functions as an anti-catastrophic factor [106]. DCX binds directly to tubulin dimers or microtubules through the two tubulin-binding domains (N-DC and C-DC) which are evolutionarily conserved tandem repeat domains. Mutations in the DCX gene in Lissencephaly patients are mostly identified as single-amino-acid substitutions within this domain region [101]. It has been shown that DCX microtubule interaction is modulated through DCX phosphorylation in the axonal wrist [107]. Phosphorylation of DCX by Cdk5 results in a free DCX whereas the dephosphorylated form of DCX by spinophilin accompanied with pp1 increases the affinity to the microtubule and leads to binding and stabilisation of the microtubule and promoting its polymerisation. DCX also interacts with dynein and LIS1 to mediate nucleus-centrosome coupling during migration [108].

Besides two microtubule binding sites, DCX consists of a Ser/Pro-rich C-terminal domain which is not required for microtubule binding [109]. This domain gives DCX a phosphoprotein characteristic containing multiple phosphorylation sites that interact with clathrin-associated proteins and several protein kinases such as the JNK, PKA, and Cdk5. Apart from the established role of DCX in microtubule dynamics, it has been suggested that DCX could play a role in vesicle trafficking via the cell adhesion molecule neurofascin. Independent of microtubule binding, DCX functions as an endocytic co-adaptor to modulate the neurofascin localisation on the plasma membrane and increase the endocytosis in neuronal culture [110]. This regulation besides other involved adaptors and molecules in endocytic membrane traffic is proposed to be crucial for neuronal migration guidance and therefore important in the development of the nervous system [111].

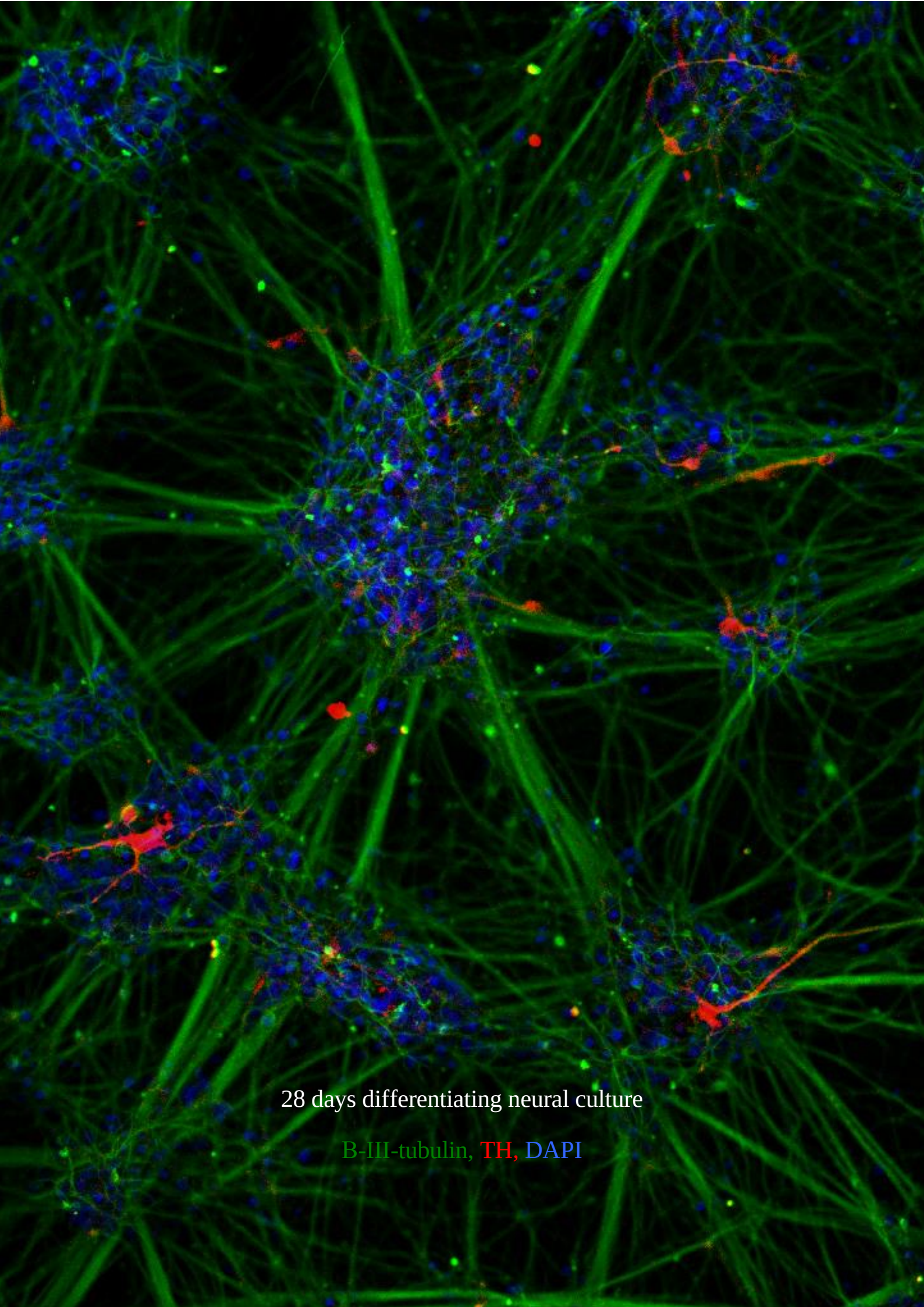
Previous studies investigating the role of DCX particularly in Lissencephaly have been performed in animal models as well as primary cell lines. The first Dcx knock-out animal model study showed remarkably normal overall brain morphology in Dcx mutant mice. The hippocampus showed disrupted lamination resulting in learning deficits despite the normal patterns of neocortical neurogenesis and neuronal migration [112]. This observation was later

shown to be due to the doublecortin-like proteins compensatory mechanism in mice [113], [114]. In contrast, a rat model using *in-utero* RNAi method demonstrated the development of an SBH like phenotype showing radial migration defects in some neuronal progenitor cells in rat neocortex. These contrasting results presented a more complicated role of DCX than current information suggests [115].

More recent studies have suggested that DCX is possibly involved in cell proliferation. Forebrain human neural progenitors derived from H9 hESC line were transfected with binary *piggyBac* transposons and then migration was followed on matrigel substrate and brain slice cultures. It was shown that ectopic expression of DCX in the forebrain human neural progenitors inhibited neural progenitor proliferation and promoted migration [116].

Animal models from recent literature theorise that double cortex formation is not only derived by neuroblast migratory defects. A GTPase RhoA mutant in the dorsal telencephalon of mice exhibited radial glial scaffold defects leading to a double cortex-like phenotype. Depletion of RhoA caused disruptions in the radial glial scaffold with loss of adherens junction anchoring and defects in the maintenance or formation of radial processes [117]. Another study suggested a different mechanism of double cortex formation. A mutation in adhesion molecules afadin or CDH2 in the dorsal telencephalon of mice drastically increased cell proliferation of progenitors that led to double cortex like phenotype. Disruption of essential adhesion junction proteins for radial glial cells significantly affected the neocortex development through altering progenitor proliferation along with abnormal axonal projections. Radial glia disruption affected cell positioning, alters progenitor proliferation, and perturbs neocortical cell layers [118].

Along with DCX, additional mechanisms have been described for guiding the migrating neurons and neurites to their targets in the brain, including the Slit/Robo signalling [119]. Slit ligands are multifunctional proteins that act importantly in a variety of developmental processes such as neurogenesis and angiogenesis [120]. One well-studied function of Slit proteins is during the development of the nervous system. Slit proteins have been shown to bind to Robo receptors and bifunctionally regulate neuronal migration and axon guidance. The chemorepulsive action of Slit proteins functioned in the regulation of migrating cells, axon guidance, and axon branching [119].



28 days differentiating neural culture

B-III-tubulin, TH, DAPI

6 KEY ASPECTS OF CHOOSING AN APPROPRIATE CELLULAR MODEL OF THE BRAIN

Several points appear to be controversial in defining a perfect cellular model. It is complex to choose a proper model that can feasibly translate results to humans and apply in clinical features. The history of the disease being studied such as heritability, sporadic or familial, or multifactorial, can influence results and make the identification of the mechanism more difficult. In the case of multifactorial diseases, large population size may help to produce more reliable data and avoid false results.

To derive a cellular model, the primary focus is on the derivation of standardised, robust, and reproducible iPSCs. How and when an iPSC line is considered a perfect line, how to decrease the heterogeneity of derived iPSC lines, and to what extent are iPSCs identical to hESCs, are still questioned. It has yet to be determined whether it is enough to show that iPSCs are similar in morphology, gene profile, and *in vivo/in vitro* differentiation capability. Alternatively, if each iPSC line should be evaluated *in vivo* and assess the responses to intrinsic signals, and observe if and how responsive they are to *in vivo* host developmental cues. To understand this better, complementary methods such as parallel experiments with hESCs or generating isogenic controls to endorse the iPSC-based model findings are required. More optimisation is required to avoid partial reprogramming and eliminate those cells which have been partially reprogrammed. Additional systematic assays might be required to thoroughly examine the iPSC differentiation potential and later on, the functionality of the differentiating cells. Is it enough to use developmental cues and generate the desired cell types, or go through the integration to the host system to evaluate the functionality? The *in vitro* link between gene expression and functionality, as well as survival and integration of derived cells to the host, remain to be shown. An important question which arises is the best way to acquire mature neurons; this might be possible with long-term culture or accelerating the differentiation by environmental factors. Perhaps multifactorial 3D platforms such as neurospheres, organoids, and microfluidic systems could provide a better environment that mimics the *in vivo* condition. 3D cultures have made notable improvements

in the field so far, and many disease features that had not been identified in monolayer cultures are on the way to recapitulation.

The epigenetic memory is a highly concerned issue. iPSCs possess specific features resulting from reprogramming itself or remaining from the original somatic cells. These newly acquired cell properties can cause complications in iPSC clinical applications. Direct reprogramming, transdifferentiation, of somatic cells to desired differentiated cell types could offer a better solution to make patient-specific cell models since the stem cell stage is skipped and they undergo a shorter reprogramming process. These cells may retain epigenetic signatures related to age which could provide more suitable cells to study neurodegenerative disease mechanisms.

To accurately reach the main goal of iPSCs for clinical application, matters discussed above must be carefully considered. Stochastic events during reprogramming and differentiation, epigenetic memory, genomic de novo mutations, prolonged cultivation of derived cells, and cell maturity, can result in significant concerns for clinical application even if this is an autologous based therapy.

There is still much to be done, even so, research is moving in the right direction to answer the questions of what is the best cellular model and how best to utilise the technique.

7 AIMS OF THE THESIS

The main goal of this doctoral thesis was to study neurological disorders and explore novel mechanisms underlying the diseases. To achieve my general goal a proper relevant cellular model was required which led me to specifically set my subgoals:

1. Derivation and establishment of human iPSC lines from healthy and diseased individuals in standardised conditions.
2. Derivation and establishment of robust, reproducible and well-defined neural progenitor populations from the healthy and patient derived-iPSC lines.
3. Identification of known disease-relevant phenotypes of Alzheimer's disease and Type 1 Lissencephaly as well as discover novel mechanisms underlying the diseases in 2D and 3D models.

8 RESULTS AND DISCUSSION

8.1 GENERATING HEALTHY AND PATIENT-SPECIFIC IPSC LINES

The first step to build an *in vitro* model was establishing induced pluripotent stem cells. We have reprogrammed patient and healthy fibroblasts at early passage by introducing Yamanaka factors Oct4, Sox2, Klf4, and c-Myc using non-integrating *Sendai virus* vectors as an efficient and reproducible system (Figure 2A-K). Transduced cells with *SeV* vectors displayed the mesenchymal to epithelial transition morphological changes from second-day post-transduction (Figure 2E). ES-like iPSC colonies were visible after 10 days. Colonies were picked manually and transferred onto irradiated human fibroblasts approximately 3 weeks post-transduction. Derived iPSCs possessed stem cell characteristics equivalent to hESCs including similar cell morphology with a large nucleus and a small cytoplasm. The colonies arose with distinct sharp edges. After reprogramming, *SeV* particles were gradually diluted out by passaging of iPSCs where they disappeared, usually by passage 10. Immunofluorescence staining confirmed expression of pluripotent stem cell markers including cell surface markers TRA1-60 and TRA1-81 and pluripotent cell nuclei markers Oct4 and Nanog. To validate the pluripotency of derived iPSC lines we chose PluriTest. iPSCs transcriptome data of an Illumina Gene Expression HT12 Direct Hybridization assay were evaluated online by uploading to the PluriTest web-portal (www.pluritest.org). The iPSC lines used in **all papers** have passed the PluriTest with low novelty indicating high pluripotency of the derived iPSC lines. Derived lines have been karyotyped with the G-banding method and showed normal 46 karyotypes. Together, all data show that the derived iPSC lines from patient and control expressed pluripotent markers and were capable of differentiating into three different germ layers while having a normal karyotype and maintaining their proliferative trait in the culture without differentiating. We have not detected any differences in the characterisation of derived lines. The data suggested that we had true iPSC lines that behaved similarly regardless of different genetic backgrounds, which allowed us to use them for the downstream aims.

In **paper I** we have shown an example of how to optimise the culture condition. Derived iPSC lines have been cultured further using recombinant spider silk, 4RepCT matrices, as a defined feeder-free system. The bio-inspired polymers from spider silk can be produced in a recombinant manner in several variants fused with known cell binding peptides from matrix proteins. The recombinant miniature spider silk protein 4RepCT fused with vitronectin is processed into 2D film or 3D foam and fibre structures. Cultivation of the healthy iPSC lines (Ctrl 3 and Ctrl 5) and hESC lines (H9, HS360, and HS181) on 4RepCT film possessed the typical stem cell morphology. Immunofluorescence analysis showed the expression of stem cell nuclei markers Oct4 and Nanog, and cell surface markers SSEA-4, TRA1-60, and TRA1-81 by flow cytometry analysis. Taken together, we have shown that the recombinant miniature spider silk can provide a xeno-free culture supporting pluripotent stem cell growth comparable to matrigel along with forming a 3D matrix and compatibility with the host immune system. Besides providing a suitable xeno-free culture coating for iPSCs, spider silk protein is biodegradable after implantation [121]. Spider silk protein can also be modified to hold recombinant proteins such as growth factors like FGF2, possibly providing a more stable and consistent way to present such factors to the cells. This can provide an optimal routine culture system for pluripotent stem cells and future iPSC based therapies.

Based on recent studies using laminin as a feeder-free coating [122], [123], we have evaluated recombinant laminin-521 for iPSC line culture and found that it is also a biologically relevant feeder-free xeno-free substrate that is suitable for stem cell culture [124].

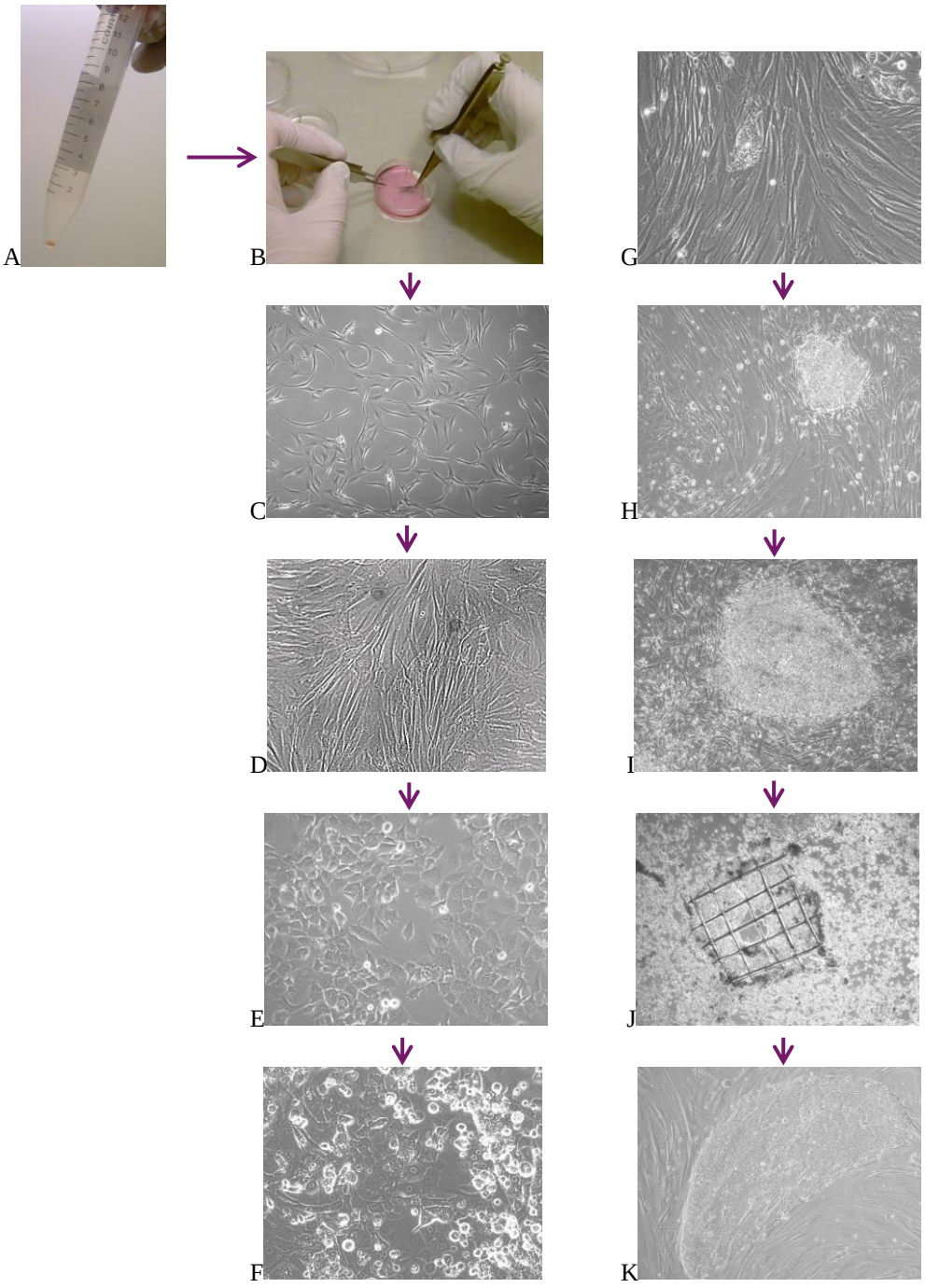


Figure 2. Derivation of hiPSCs. (A-C) Patient and healthy fibroblasts are established from a skin biopsy. (D) Fibroblasts at a low passage with 70-80% confluency are induced with Yamanaka factors. Morphological changes are seen in transfected cells 2 (E) and 6 (F) days post-transduction. (G-I) Emerged iPSC colony one (G), two (H) and three (I) weeks post-transduction (J) Colonies are manually picked and transferred to inactivated HFFs. (K) A typical iPSC colony on inactivated HFFs.

8.2 GENERATING WELL-DEFINED NEURAL PROGENITOR POPULATIONS FOR *IN VITRO* STUDIES OF NEUROLOGICAL DISORDERS

Direct differentiation of ESCs and iPSCs to the desired neurons has generally been a long process resulting in a heterogeneous neuronal population consisting of unwanted cell types. We sought to generate intermediate expandable NESC lines from patients and healthy undiagnosed individuals. All NESC lines in the study have been generated in the lab based on protocols we have derived and modified in **Paper III** and [70] (Figure 3). Morphologically, the derived NESCs self-organised into rosette structures with a mermaid shape that grew head to head. They expressed neural precursor markers Nestin and SOX2, SOX1 and PAX6. Derived NESCs were also positive for rosette marker transcription factors PLZF and DACH1 as well as the polarity marker of neuroepithelial ZO-1. In **paper III** transcriptome data analysis of different NESC lines from two Lissencephaly patients and two controls represented high similarity between lines. Hierarchical clustering showed the NESCs clustered together separate from the differentiating cells. The expression of general neural progenitor markers of the DCX mutant and control derived NESC lines such as Nestin, SOX2, SOX1, PLZF, DACH1, PAX6, HES5, and MMNR1 did not differ at NESC stage. This transcriptional similarity proposed the homogeneity of our NESC population despite patient genomic background variation.

Our data showed that we have successfully established a robust system to generate a stable neural progenitor population that represented the *in vivo* early neural stem cell characteristics, which made them a suitable system for modelling early human neurological development in health and disease.

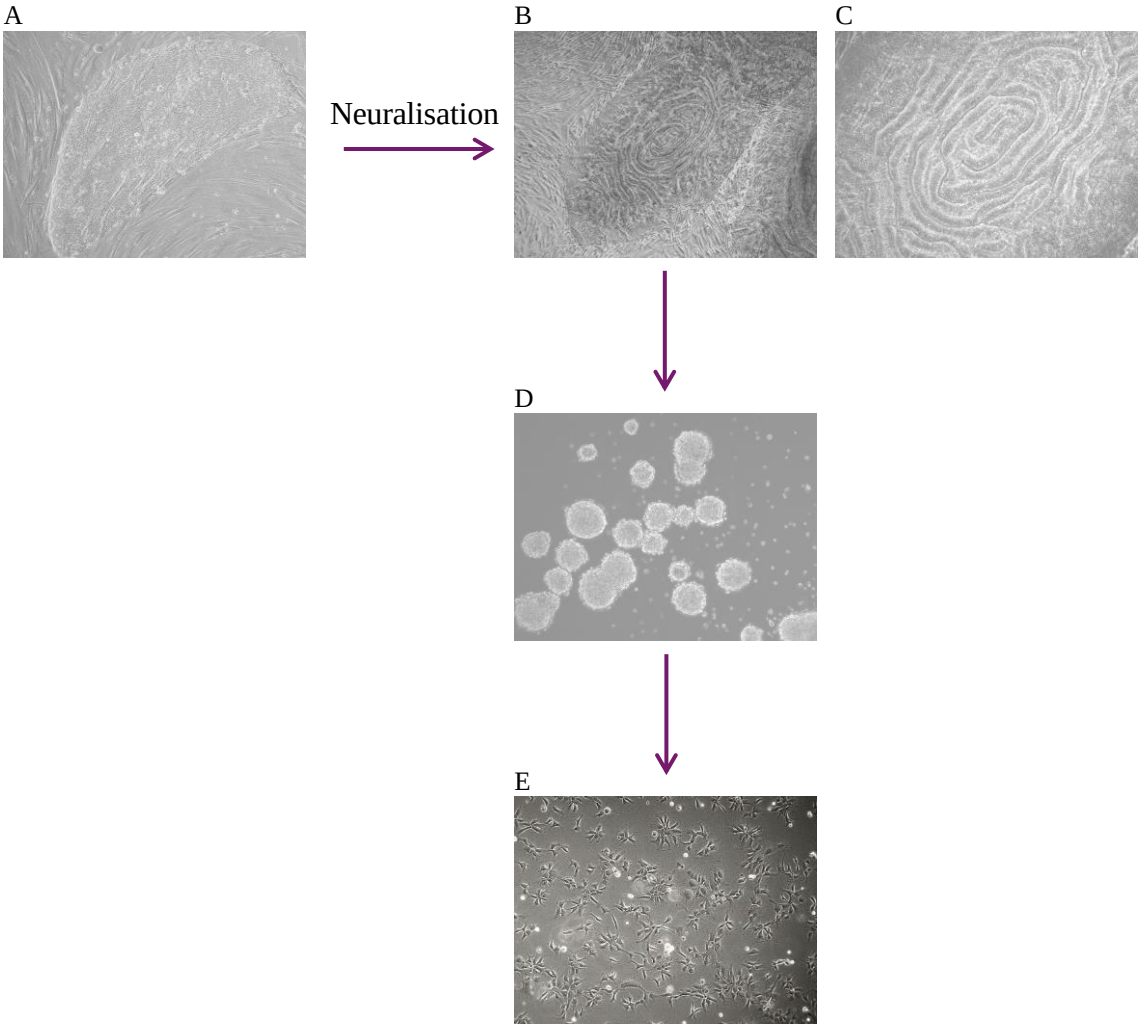


Figure 3. Generating a stable NESC line. (A) 60-70% confluent iPSC culture is neurally induced in the presence of LIF and chemically defined factors including CHIR, SB431542, and DAPT for 5 to 7 days. (B, C) Neuralisation is observed after 5 days. (D) Neural rosettes are picked manually and cultured in suspension for 48 hours. After two days, neurospheres are dissociated and (E) Single cells are plated on a poly-L-ornithine/laminin-coated plate in NESC medium.

8.3 AN APPROPRIATE CELLULAR MODEL MIMICS THE DISEASE KNOWN RELATED PHENOTYPE

8.3.1 AD model

In **paper II** we have modelled Alzheimer's disease (AD) with a healthy derived NESC line to explore the disease-related phenotype in the dish. NESCs have successfully been differentiated to cholinergic neuronal subtypes on 2D and further subcultured on 3D (SAP: self-assembling peptide matrix) with the sequential application of extrinsic factors Shh, BMP9, and NGF which supports cholinergic neurons in the forebrain. First, we assessed the effect of two culture conditions on expression patterns of some AD-associated proteins including pPAK, Cofilin, and Drebrin in differentiated cells. Quantitative immunofluorescence analysis revealed significant differences between 2D and 3D cultivation, which suggested that neurons in 3D culture more closely mimicked the *in vivo* situation compared to a conventional 2D culture. Both 2D and 3D cultures were then treated with beta-amyloid (Aβ) oligomers to mimic the AD pathology in the dish. Interestingly, the disease-related phenotypes, reduction of pPAK and Drebrin protein levels, could only be recapitulated in 3D culture and not in standard 2D culture when treated with Aβ oligomers. Our data has shown that Aβ oligomers in 3D culture affected the subcellular localisation of pPAK and Drebrin proteins from cytosolic and nucleus regions to sub-membranous regions, which have also been seen *in vivo* in post-mortem Alzheimer's brains [125]. P21-activated kinase proteins (PAKs) are involved in brain diseases, particularly AD, functioning on neuronal morphology as well as forebrain development [126]. It has been reported that the PAKs and Drebrin levels are reduced in AD patient brains, at the protein level and even more so on the activated protein levels; pPAK. This finding is in accordance with animal models and AD patient brains. These results show the unique potential of NESCs cultured in 3D to faithfully model human disease and being a suitable tool for *in vitro* disease modelling.

8.3.2 Type 1 Lissencephaly model

In **paper III** we studied Lissencephaly using iPSC-derived neuroepithelial stem cells from two patients carrying Type 1 Lissencephaly. The first patient (DCX-R19Stop) carries a nonsense mutation (c.C55T, p.Arg19ATOP) and the second patient (DCX-T88M) a missense mutation (c.C263T, pThr88Met), both male. To derive disease-relevant cell types, we have reprogrammed fibroblast cells from patients and two samples from healthy persons using *SeV*. Characterized iPSC lines were neurally induced to generate NESCs. Derived patients NESCs did not indicate any phenotype differences regarding the expression of neural stem cell markers as well as proliferation rate.

8.3.2.1 Patient cells exhibited different DCX expression patterns compared to control cells upon differentiation

To reveal the DCX expression profile, NESC lines were differentiated spontaneously to neurons by removing growth factors EGF and bFGF from the medium for 21 days. Immunofluorescence analysis indicated that mutant and control NESCs at proliferative stage did not express any DCX, whereas, upon differentiation, the expression was massively upregulated only in control cells, both on the RNA and protein levels. In contrast, DCX-R19Stop cells did not show any detectable DCX protein expression in differentiating neuroblasts and DCX-T88M cells expressed a slight amount of protein with more localization at the soma. DCX-R19Stop was derived from a patient with a premature stop codon at amino acid position 19 at the beginning of the protein while DCX-T88M has an amino acid substitution at position 88, which is in one of the microtubule binding domains. This could be an explanation why DCX protein is not detectable in DCX-R19Stop.

Transcriptome data analysis of NESCs and differentiating cells from patients and controls revealed the effect of the DCX expression patterns. Hierarchical cluster diagram of transcriptome data analysis revealed that at NESC stage when the DCX protein is not expressed, NESCs from patients and controls clustered together separate from differentiating cells. Upon differentiation when DCX expression is upregulated, patient cells clustered together separate from control cells. This analysis indicated that deregulation of DCX in patient

cells directly affected the gene expression profile pattern and caused this clustering and separation. Based on the data we decided to look more closely only at diff control and diff patient cells (diff: differentiation day 7 to day21) increasing the statistical power. We have applied the $p<0.05$ cutoff and observed a large number of differentially expressed genes, being either up-regulated (4367) or down-regulated (2421) in patient differentiating cells. These huge transcriptional differences appeared to affect several phenomena such as migration and neurite outgrowth.

8.3.2.2 DCX upregulation is connected with several phenomena during neuronal differentiation

We have examined the neuronal migration capability of patient and control neuroblasts during differentiation. Results from clonal migration assay and time-lapse microscopy revealed a clear migration phenotype of mutated cells at both the pre-differentiated and differentiating stages; the methods are well-described in **paper III**. Time-lapse results of plated neurospheres over one-week pre-differentiation indicated less migration in DCX mutant compared to control. Furthermore, results from the clonal migration assay over a longer time of three weeks differentiation displayed a significant difference in patient and control cells. Again, DCX mutant cells migrated less and seemed to prefer to stay in the plating site rather than migrating. With this, we provided evidence to support the fact that Lissencephaly is considered as a migrational disorder.

Additionally, we have observed fewer and thinner dendrite bundles, stained with neuronal marker MAP2, in differentiating neuronal culture in patient cells compared to control. We have also looked at neurite extension; the first stage which takes place in migrating neurons followed by translocation of the nucleus and cytoplasmic components [127]. Cells in the pre-differentiation stage were dissociated and cultured in suspension for 24 hours to form neurospheres. Neurospheres were selected and embedded into matrigel on a cover glass. Emerged neurite length of differentiating neurospheres of patient and control samples were measured the following day using ImageJ. A shorter neurite length was observed in both patient lines. It has been reported that DCX is involved in neurite outgrowth through the JNK pathway

[127], [128]. JNK phosphorylates the DCX and stimulates neurite outgrowth. The shorter neurite length in patient cells might be a result of the defect in phosphorylation of DCX by JNK, which infers that the part of the protein fulfilling a function in neurite outgrowth is either not existing or not properly functioning in mutated cells.

Data from transcriptome analysis supported the observed phenotypes. Hierarchical clustering and PCA analysis both indicated that patient cells cluster together away from control cells based on differentiation status. Interestingly, we identified miss-regulation of genes that are involved in cell migration, neurite outgrowth, and synapse maturation such as NTNG1, CHL1, and PPP1R9A which was consistent with the observed phenotype. We remarked some of the top scoring genes such as RELN, CNTN1, GABRR1, and FABP7 are disease-associated and have been reported in human brain disorders [129], [130], [131], [132]. Presence of cadherin superfamily members among differentially expressed genes suggested a probable defect in crosstalk between cell-cell adhesions and that could be one of the causes of defective migration. We continued with GO analysis and found out the involvement of highly relevant biological processes including neuronal differentiation, migration, axonal outgrowth and unexpectedly, cell proliferation.

Our data is suggesting that the lack of a functional DCX protein can interfere with neurogenesis and lead to migrational defects, disrupt the neurite growth process, and fewer and thinner dendrite bundles in differentiating patient cells. This proposes an important role of DCX up-regulation when progenitor neurons initiate differentiation and migration.

8.3.2.3 SLIT3 knockdown and treatment with recombinant Robo1/Robo2 proteins rescued the shorter neurites phenotype exhibited by Lissencephaly patients' cells

We have taken advantage of our transcriptome data and looked closely at the differentially expressed genes to investigate the possible cause of the observed phenotype of the shorter neurite length in patients' neuroblasts. Based on literature and regarding the already known associated genes in migration and neurite outgrowth, we found that the gene SLIT3 was among the up-regulated genes in patient neuronal cells during differentiation. We attempted to generate SLIT3 knockdown NESC lines by using shRNA construct. Then performed neurite outgrowth assay with the SLIT3 knockdown patient and control NESC lines. SLIT3 depletion rescued the detected phenotype; patients' neurospheres from SLIT3 shRNA knockdown lines displayed significantly longer neurite length.

In a parallel neurite outgrowth experiment, we applied the SLIT receptors to investigate whether the SLIT inhibition is mediating the neurite outgrowth extension. Data demonstrated that the exogenous application of Robo occupied the ligands preventing the SLIT3 protein from binding to the endogenous Robo receptors presented in neurospheres. By this, neurite outgrowth of DCX-R19Stop and DCX-T88M was no longer suppressed.

8.3.2.4 Unexpected phenotype of Lissencephaly: DCX mutated cells exhibited a prolonged proliferation phenotype upon leaving NESC stage.

Upon growth factor withdraw we have noticed patients NESCs remain proliferative. To evaluate the observed phenotype, we counted the number of cells as well as the number of Ki67 positive cells during the differentiation window. NESCs were spontaneously differentiated in 24 well plates by removing growth factors bFGF and EGF from the medium. Cells were counted every third day to follow the proliferation rate. Proliferation gradually ceased in healthy NESCs by growth factor removal; cells began to differentiate, become postmitotic, and proceeded into maturation. DCX-mutant cell counts were almost twice that of control cells following two weeks of differentiation. At the beginning of differentiation, more than 90% of patient and control cells stained positive for the proliferative marker Ki67. Upon differentiation, up to 70 % of control cells lost the Ki67 marker whereas DCX mutated cells maintained a higher percentage, 55% of the cells were still Ki67 positive.

The patient neural stem cells seemed somewhat resistant to differentiation, suggesting that the lack of functional DCX protein in patient cells may have forced them to remain in the stem-cell state, rather than to differentiate into new neuronal identities. Additionally, data from transcriptome analysis supported the observed phenotype. Genes connected to proliferation and cell adhesion remained upregulated in patient cells during differentiation compared to healthy cells.

9 CONCLUSIONS AND FUTURE PERSPECTIVES

The main goal of this doctoral thesis was to study neurological disorders and explore novel mechanisms underlying the diseases. As discussed in the beginning, it is difficult to study the early stage of human development *in vivo* for several reasons. Animal models have commonly been used as a research model to study human diseases, and although these animal models have contributed significantly to the research revealing valuable information, it is still challenging to extrapolate results obtained from animal experiments to humans. Therefore, in the absence of a comparable model organism, a suitable alternative human system is demanded. Regarding the ethical issues, a human cellular *in vitro* system could serve scientists as a prominent tool to study human disease mechanisms. iPSC technology has opened a new window in investigating underlying disease mechanisms and overcoming the above issues. iPSC technology allows scientists to rely on data from using *in vitro* models more safely. A consistent system in a standardised condition is essential to limit variations and obtain more reliable data that can be applied in the future for therapeutic applications and regenerative medicine. To identify brain disease-relevant mechanisms, it is crucial to be able to compare NESC derived neurons from genetically distinct pluripotent cells in a consistent manner. To do this, we need to produce a robust derivation and cultivation system from pluripotent cells to neurons, with high reproducibility that provides us with stable and comparable well-characterised cell lines.

The results presented in **paper I** showed an example of standardisation of culture conditions. We showed that recombinant spider silk supported pluripotent stem cells growth while maintaining pluripotency as well as differentiation potential into three germ layers. Recombinant spider silk could be an example of an optimal routine xeno-free culture in both 2D and 3D systems for pluripotent stem cells.

After derivation and characterisation of iPSC lines, we then attempted generating NESCs. Here we have established a reliable *in vitro* method to generate and differentiate NESC lines from patient and healthy samples. We have shown that NESC populations derived from healthy and disease donors maintained a homogenous population, they showed similarity in gene expression profile despite their different genetic backgrounds.

In **paper II** we used a control NESC line to model Alzheimer's disease. We showed the potential of our healthy derived NESC line as a suitable tool to model AD and explore the

disease-related phenotype in the dish. Recapitulating AD-related phenotypes only in 3D neuronal culture suggested the importance of environmental context in cellular disease modelling. We proposed that 3D models which closely mimic the *in vivo* situation can be employed as more refined tools for studying brain development and disorders.

Finally, we have used patient-specific derived NESCs to investigate Type 1 Lissencephaly phenotype in a dish. Data presented in **paper III** confirmed the known migratory deficit in patient DCX mutant neuroblasts. The migration defects combined with the apparent initial resistance of patient NESCs to differentiate and aberrant neurite outgrowth suggested a central role of DCX up-regulation when progenitor neurons initiate differentiation and later migration. Interestingly, our data revealed that the two different DCX mutations exhibited the same phenotypes. Based on literature research, this study is the first cellular model of Lissencephaly using patient DCX mutant iPSCs.

Further investigation needs to be done to explore the exact role of DCX along with which cellular signals and/or downstream effectors that are terminating NESC proliferation and initiating differentiation and migration. Future work would involve being able to compare a homogeneous neuroblast cell population. Looking at different stages of differentiation to neurons would also be important in creating a robust method. One way would be to sort cells or use reporter constructs to obtain a more homogenous population. For example, using a fluorescent reporter under the control of the human DCX promoter to ensure the comparison of only DCX expressing cells from patient and control. Cell sorting would be an advantage when studying migration for instance, where we can track the same cell population. And finally, using the genome editing technology, the RNA-guided nucleases (CRISPR/CAS9), to correct the mutation and attempt to rescue the phenotype which in turns offer a unique platform in future for personalised therapies and drug screening.

This thesis underlines the importance of a cellular model to study human brain development and disease. We indicate that it is possible to develop a reliable *in vitro* model of both neurodevelopmental and neurodegenerative disorders to verify the disease phenotypes and uncover underlying mechanisms in 2D and 3D systems. This cellular model could offer novel approaches to therapeutic applications of complex neurological diseases taking scientists one step closer to treatment.

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11 REFERENCES

[1] M. J. Evans and M. H. Kaufman, “Establishment in culture of pluripotential cells from mouse embryos.,” *Nature*, vol. 292, no. 5819. pp. 154–156, 1981.

[2] G. R. Martin, “Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.,” *Proc. Natl. Acad. Sci.*, vol. 78, no. 12, pp. 7634–7638, 1981.

[3] J. a Thomson, J. Itskovitz-Eldor, S. S. Shapiro, M. a Waknitz, J. J. Swiergiel, V. S. Marshall, and J. M. Jones, “Embryonic stem cell lines derived from human blastocysts.,” *Science*, vol. 282, no. 5391, pp. 1145–1147, 1998.

[4] R. Eiges, A. Urbach, M. Malcov, T. Frumkin, T. Schwartz, A. Amit, Y. Yaron, A. Eden, O. Yanuka, N. Benvenisty, and D. Ben-Yosef, “Developmental Study of Fragile X Syndrome Using Human Embryonic Stem Cells Derived from Preimplantation Genetically Diagnosed Embryos,” *Cell Stem Cell*, vol. 1, no. 5, pp. 568–577, 2007.

[5] A. Urbach, M. Schuldiner, and N. Benvenisty, “Modeling for Lesch-Nyhan disease by gene targeting in human embryonic stem cells.,” *Stem Cells*, vol. 22, no. 4, pp. 635–41, 2004.

[6] K. Takahashi and S. Yamanaka, “Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors,” *Cell*, vol. 126, no. 4, pp. 663–676, Aug. 2006.

[7] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka, “Induction of pluripotent stem cells from adult human fibroblasts by defined factors.,” *Cell*, vol. 131, no. 5, pp. 861–72, Nov. 2007.

[8] J. Yu, M. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian, J. Nie, G. a Jonsdottir, V. Ruotti, R. Stewart, I. I. Slukvin, and J. a Thomson, “Induced pluripotent stem cell lines derived from human somatic cells.,” *Science (80-.).*, vol. 318, no. 5858, pp. 1917–20, 2007.

[9] S. S. W. Han, L. a Williams, and K. C. Eggan, “Constructing and deconstructing stem cell models of neurological disease.,” *Neuron*, vol. 70, no. 4, pp. 626–44, May 2011.

[10] M. Bellin, M. C. Marchetto, F. H. Gage, and C. L. Mummery, “Induced pluripotent stem cells: the new patient?,” *Nat. Rev. Mol. Cell Biol.*, vol. 13, no. 11, pp. 713–26, Nov. 2012.

[11] S. Tamir Rashid and G. J. M. Alexander, “Induced pluripotent stem cells: from nobel prize to clinical applications.,” *J. Hepatol.*, vol. null, no. null, Nov. 2012.

[12] D. a Robinton and G. Q. Daley, “The promise of induced pluripotent stem cells in research and therapy.,” *Nature*, vol. 481, no. 7381, pp. 295–305, Jan. 2012.

[13] G. Amabile and A. Meissner, “Induced pluripotent stem cells: current progress and potential for regenerative medicine.,” *Trends Mol. Med.*, vol. 15, no. 2, pp. 59–68, Feb. 2009.

[14] M. Lin, E. Pedrosa, A. Shah, A. Hrabovsky, S. Maqbool, D. Zheng, and H. M. Lachman, “RNA-Seq of human neurons derived from iPS cells reveals candidate long non-coding RNAs

involved in neurogenesis and neuropsychiatric disorders.," *PLoS One*, vol. 6, no. 9, p. e23356, Jan. 2011.

[15] B. A. DeRosa, J. M. Van Baaren, G. K. Dubey, J. M. Lee, M. L. Cuccaro, J. M. Vance, M. A. Pericak-Vance, and D. M. Dykxhoorn, "Derivation of autism spectrum disorder-specific induced pluripotent stem cells from peripheral blood mononuclear cells.," *Neurosci. Lett.*, vol. 516, no. 1, pp. 9–14, May 2012.

[16] S. P. Paşca, T. Portmann, I. Voineagu, M. Yazawa, A. Shcheglovitov, A. M. Paşca, B. Cord, T. D. Palmer, S. Chikahisa, S. Nishino, J. A. Bernstein, J. Hallmayer, D. H. Geschwind, and R. E. Dolmetsch, "Using iPSC-derived neurons to uncover cellular phenotypes associated with Timothy syndrome," *Nature Medicine*, vol. 17, no. 12. pp. 1657–1662, 2011.

[17] A. Urbach, O. Bar-Nur, G. Q. Daley, and N. Benvenisty, "Differential modeling of fragile X syndrome by human embryonic stem cells and induced pluripotent stem cells," *Cell Stem Cell*, vol. 6, no. 5, pp. 407–411, 2010.

[18] O. Bar-Nur, I. Caspi, and N. Benvenisty, "Molecular analysis of FMR1 reactivation in fragile-X induced pluripotent stem cells and their neuronal derivatives.," *J. Mol. Cell Biol.*, vol. 4, no. 3, pp. 180–3, Jun. 2012.

[19] M. C. N. Marchetto, C. Carromeu, A. Acab, D. Yu, G. W. Yeo, Y. Mu, G. Chen, F. H. Gage, and A. R. Muotri, "A model for neural development and treatment of rett syndrome using human induced pluripotent stem cells," *Cell*, vol. 143, no. 4, pp. 527–539, 2010.

[20] X. Mou, Y. Wu, H. Cao, Q. Meng, Q. Wang, C. Sun, S. Hu, Y. Ma, and H. Zhang, "Generation of disease-specific induced pluripotent stem cells from patients with different karyotypes of Down syndrome," *Stem Cell Research & Therapy*, vol. 3, no. 2. p. 14, 2012.

[21] J. P. Weick, D. L. Held, G. F. Bonadurer, M. E. Doers, Y. Liu, C. Maguire, A. Clark, J. a Knackert, K. Molinarolo, M. Musser, L. Yao, Y. Yin, J. Lu, X. Zhang, S.-C. Zhang, and A. Bhattacharyya, "Deficits in human trisomy 21 iPSCs and neurons.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 24, pp. 9962–7, 2013.

[22] T. Yagi, D. Ito, Y. Okada, W. Akamatsu, Y. Nihei, T. Yoshizaki, S. Yamanaka, H. Okano, and N. Suzuki, "Modeling familial Alzheimer's disease with induced pluripotent stem cells.," *Hum. Mol. Genet.*, vol. 20, no. 23, pp. 4530–9, Dec. 2011.

[23] M. a Israel, S. H. Yuan, C. Bardy, S. M. Reyna, Y. Mu, C. Herrera, M. P. Hefferan, S. Van Gorp, K. L. Nazor, F. S. Boscolo, C. T. Carson, L. C. Laurent, M. Marsala, F. H. Gage, A. M. Remes, E. H. Koo, and L. S. B. Goldstein, "Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells.," *Nature*, vol. 482, no. 7384, pp. 216–20, Feb. 2012.

[24] J. T. Dimos, K. T. Rodolfa, K. K. Niakan, L. M. Weisenthal, H. Mitsumoto, W. Chung, G. F. Croft, G. Saphier, R. Leibel, R. Goland, H. Wichterle, C. E. Henderson, and K. Eggan, "Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. TL - 321," *Science*, vol. 321 VN-, no. 5893, pp. 1218–1221, 2008.

[25] N. Ichiyanagi, K. Fujimori, M. Yano, C. Ishihara-Fujisaki, T. Sone, T. Akiyama, Y. Okada, W. Akamatsu, T. Matsumoto, M. Ishikawa, Y. Nishimoto, Y. Ishihara, T. Sakuma, T. Yamamoto, H. Tsuiji, N. Suzuki, H. Warita, M. Aoki, and H. Okano, "Establishment of in Vitro FUS-

Associated Familial Amyotrophic Lateral Sclerosis Model Using Human Induced Pluripotent Stem Cells," *Stem Cell Reports*, vol. 6, no. 4, pp. 496–510, 2016.

[26] S. T. Rashid and G. J. M. Alexander, "Induced pluripotent stem cells: From Nobel Prizes to clinical applications," *J. Hepatol.*, vol. 58, no. 3, pp. 625–629, 2013.

[27] L. David and J. M. Polo, "Phases of reprogramming," *Stem Cell Res.*, vol. 12, no. 3, pp. 754–761, 2014.

[28] A. Soufi, G. Donahue, and K. S. Zaret, "Facilitators and impediments of the pluripotency reprogramming factors' initial engagement with the genome," *Cell*, vol. 151, no. 5, pp. 994–1004, 2012.

[29] R. Sridharan, J. Tchieu, M. J. Mason, R. Yachechko, E. Kuoy, S. Horvath, Q. Zhou, and K. Plath, "Role of the murine reprogramming factors in the induction of pluripotency.," *Cell*, vol. 136, no. 2, pp. 364–77, Jan. 2009.

[30] D. Subramanyam, S. Lamouille, R. L. Judson, J. Y. Liu, N. Bucay, R. Derynck, and R. Blelloch, "Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells," *Nat. Biotechnol.*, vol. 29, no. 5, pp. 443–448, 2011.

[31] L. S. Ying SY., Fang W., "The miR-302-Mediated Induction of Pluripotent Stem Cells (iPSC): Multiple Synergistic Reprogramming Mechanisms.," *Methods Mol. Biol.*, vol. 1733, pp. 283–304, 2018.

[32] F.-J. Müller, B. M. Schuldt, R. Williams, D. Mason, G. Altun, E. P. Papapetrou, S. Danner, J. E. Goldmann, A. Herbst, N. O. Schmidt, J. B. Aldenhoff, L. C. Laurent, and J. F. Loring, "A bioinformatic assay for pluripotency in human cells.," *Nat. Methods*, vol. 8, no. 4, pp. 315–7, Apr. 2011.

[33] K. Okita, T. Ichisaka, and S. Yamanaka, "Generation of germline-competent induced pluripotent stem cells," *Nature*, vol. 448, no. 7151, pp. 313–317, 2007.

[34] N. Maherali, R. Sridharan, W. Xie, J. Utikal, S. Eminli, K. Arnold, M. Stadtfeld, R. Yachechko, J. Tchieu, R. Jaenisch, K. Plath, and K. Hochedlinger, "Directly Reprogrammed Fibroblasts Show Global Epigenetic Remodeling and Widespread Tissue Contribution," *Cell Stem Cell*, vol. 1, no. 1, pp. 55–70, 2007.

[35] S. Yamanaka, "Induced pluripotent stem cells: Past, present, and future," *Cell Stem Cell*, vol. 10, no. 6, pp. 678–684, 2012.

[36] N. Fusaki, H. Ban, A. Nishiyama, K. Saeki, and M. Hasegawa, "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," *Proc. Japan Acad. Ser. B*, vol. 85, no. 8, pp. 348–362, 2009.

[37] C. C. Macarthur, A. Fontes, N. Ravinder, D. Kuninger, J. Kaur, M. Bailey, A. Taliana, M. C. Vemuri, and P. T. Lieu, "Generation of human-induced pluripotent stem cells by a nonintegrating RNA Sendai virus vector in feeder-free or xeno-free conditions.," *Stem Cells Int.*, vol. 2012, p. 564612, Jan. 2012.

[38] H. O. Li, Y. F. Zhu, M. Asakawa, H. Kuma, T. Hirata, Y. Ueda, Y. S. Lee, M. Fukumura, a Iida, a Kato, Y. Nagai, and M. Hasegawa, "A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression.," *J. Virol.*, vol. 74, no. 14, pp. 6564–9, Jul. 2000.

[39] Y. Yonemitsu, C. Kitson, S. Ferrari, R. Farley, U. Griesenbach, D. Judd, R. Steel, P. Scheid, J. Zhu, P. K. Jeffery, A. Kato, M. K. Hasan, Y. Nagai, I. Masaki, M. Fukumura, M. Hasegawa, D. M. Geddes, and E. W. Alton, "Efficient gene transfer to airway epithelium using recombinant Sendai virus.," *Nat. Biotechnol.*, vol. 18, no. 9, pp. 970–973, 2000.

[40] S. Ferrari, U. Griesenbach, A. Iida, R. Farley, A. M. Wright, J. Zhu, F. M. Munkonge, S. N. Smith, J. You, H. Ban, M. Inoue, M. Chan, C. Singh, B. Verdon, B. E. Argent, B. Wainwright, P. K. Jeffery, D. M. Geddes, D. J. Porteous, S. C. Hyde, M. A. Gray, M. Hasegawa, and E. W. F. W. Alton, "Sendai virus-mediated CFTR gene transfer to the airway epithelium.," *Gene Ther.*, vol. 14, no. 19, pp. 1371–1379, 2007.

[41] A. Takeda, H. Igarashi, M. Kawada, T. Tsukamoto, H. Yamamoto, M. Inoue, A. Iida, T. Shu, M. Hasegawa, and T. Matano, "Evaluation of the immunogenicity of replication-competent V-knocked-out and replication-defective F-deleted Sendai virus vector-based vaccines in macaques," *Vaccine*, vol. 26, no. 52, pp. 6839–6843, 2008.

[42] F. Anokye-danso, C. M. Trivedi, D. Juhr, M. Gupta, Z. Cui, Y. Tian, Y. Zhang, W. Yang, P. J. Gruber, J. A. Epstein, and E. E. Morrisey, "Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency," *Cell Stem Cell*, vol. 8, no. 4, pp. 376–388, 2011.

[43] N. Miyoshi, H. Ishii, H. Nagano, N. Haraguchi, D. L. Dewi, Y. Kano, S. Nishikawa, M. Tanemura, K. Mimori, F. Tanaka, T. Saito, J. Nishimura, I. Takemasa, T. Mizushima, M. Ikeda, H. Yamamoto, M. Sekimoto, Y. Doki, and M. Mori, "Reprogramming of mouse and human cells to pluripotency using mature microRNAs," *Cell Stem Cell*, vol. 8, no. 6, pp. 633–638, 2011.

[44] L. Warren, P. D. Manos, T. Ahfeldt, Y. H. Loh, H. Li, F. Lau, W. Ebina, P. K. Mandal, Z. D. Smith, A. Meissner, G. Q. Daley, A. S. Brack, J. J. Collins, C. Cowan, T. M. Schlaeger, and D. J. Rossi, "Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA," *Cell Stem Cell*, vol. 7, no. 5, pp. 618–630, 2010.

[45] J. Yu, K. Hu, K. Smuga-Otto, S. Tian, R. Stewart, I. I. Slukvin, and J. A. Thomson, "Human Induced Pluripotent Stemm Cells Free of Vector and Trangene Sequence," *Science (80-.).*, vol. 324, no. June, pp. 797–802, 2009.

[46] K. Okita, Y. Matsumura, Y. Sato, A. Okada, A. Morizane, S. Okamoto, H. Hong, M. Nakagawa, K. Tanabe, K. I. Tezuka, T. Shibata, T. Kunisada, M. Takahashi, J. Takahashi, H. Saji, and S. Yamanaka, "A more efficient method to generate integration-free human iPS cells," *Nat. Methods*, vol. 8, no. 5, pp. 409–412, 2011.

[47] A. Kamath, S. Ternes, S. McGowan, A. English, R. Mallampalli, and A. B. M. Moy, "Efficient method to create integration- induced pluripotent stem cells from adherent cells," *Futur. Sci.*, vol. 3, 2017.

[48] D. Kim, C. H. Kim, J. Il Moon, Y. G. Chung, M. Y. Chang, B. S. Han, S. Ko, E. Yang, K. Y.

Cha, R. Lanza, and K. S. Kim, “Generation of Human Induced Pluripotent Stem Cells by Direct Delivery of Reprogramming Proteins,” *Cell Stem Cell*, vol. 4, no. 6, pp. 472–476, 2009.

[49] H. Zhou, S. Wu, J. Y. Joo, S. Zhu, D. W. Han, T. Lin, S. Trauger, G. Bien, S. Yao, Y. Zhu, G. Siuzdak, H. R. Schöler, L. Duan, and S. Ding, “Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins,” *Cell Stem Cell*, vol. 4, no. 5, pp. 381–384, 2009.

[50] C. Desponts and S. Ding, “Using Small Molecules to Improve Generation of Induced Pluripotent Stem Cells from Somatic Cells,” vol. 636, Humana Press, 2010, pp. 207–218.

[51] D. Huangfu, K. Osafune, R. Maehr, W. Guo, A. Eijkelenboom, S. Chen, W. Muhlestein, and D. A. Melton, “Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2,” *Nat. Biotechnol.*, vol. 26, no. 11, pp. 1269–1275, 2008.

[52] M. Amit, V. Margulets, H. Segev, K. Shariki, I. Laevsky, R. Coleman, and J. Itskovitz-Eldor, “Human feeder layers for human embryonic stem cells.,” *Biol. Reprod.*, vol. 68, no. 6, pp. 2150–2156, 2003.

[53] Xu C1, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, and Carpenter MK., “Feeder-free growth of undifferentiated human embryonic stem cells,” *Nat. Biotechnol*, vol. 19, no. 10, pp. 971–974, 2001.

[54] H. K. Kleinman, M. L. McGarvey, L. A. Liotta, P. G. Robey, K. Tryggvason, and G. R. Martin, “Isolation and Characterization of Type IV Procollagen, Laminin, and Heparan Sulfate Proteoglycan from the EHS Sarcomat,” *Biochemistry*, vol. 23, no. 21(24), pp. 6188–93, 1892.

[55] E. Sanjar and S. Jin, “Substrates for clinical applicability of stem cells,” *World J. Stem Cells*, vol. 7, no. 2, p. 243, Mar. 2015.

[56] M. R. Livesey, D. Magnani, G. E. Hardingham, S. Chandran, and D. J. A. Wyllie, “Functional properties of *in vitro* excitatory cortical neurons derived from human pluripotent stem cells,” *J. Physiol.*, vol. 594, no. 22, pp. 6573–6582, 2016.

[57] K. C. Vadodaria, D. N. Amatya, M. C. Marchetto, and F. H. Gage, “Modeling psychiatric disorders using patient stem cell-derived neurons: a way forward,” *Genome Med.*, vol. 10, no. 1, p. 1, 2018.

[58] B. A. Reynolds and S. Weiss, “Generation of Neurons and Astrocytes from Isolated Cells of the Adult Mammalian Central Nervous System,” *Science (80-.).*, vol. 255, no. 5052, pp. 1707–1710, 1992.

[59] A. Gritti, E. Parati, L. Cova, P. Frolichsthal, R. Galli, E. Wanke, L. Faravelli, D. Morassutti, F. Roisen, D. Nickel, and A. L. Vescovi, “Multipotential Stem Cells from the Adult Mouse and Self-Renew in Response to Basic Fibroblast,” *J. Neurosci.*, vol. 16, no. 3, pp. 1091–1100, 1996.

[60] B. Kirschenbaum, M. Nedergaard, A. Preuss, K. Barami, R. A. R. Fraser, and S. A. Goldman, “In vitro neuronal production and differentiation by precursor cells derived from the adult human forebrain,” *Cereb. Cortex*, vol. 4, no. 6, pp. 576–589, 1994.

[61] V. G. Kukekov, E. D. Laywell, O. Suslov, K. Davies, B. Scheffler, L. B. Thomas, T. F.

O'Brien, M. Kusakabe, and D. A. Steindler, "Multipotent stem/progenitor cells with similar properties arise from neurogenic regions of adult human brain," *Exp. Neurol.*, vol. 156, no. 2, pp. 333–344, 1999.

[62] Y. Arsenijevic, J. G. Villemure, J. F. Brunet, J. J. Bloch, N. Déglon, C. Kostic, A. Zurn, and P. Aebischer, "Isolation of multipotent neural precursors residing in the cortex of the adult human brain," *Exp. Neurol.*, vol. 170, no. 1, pp. 48–62, 2001.

[63] S. Okabe, F.-N. Karin, C. A. Spiro, M. Segal, and M. Ronald, "Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro," vol. 59, no. 1, pp. 89–102, 1999.

[64] T. Barberi, P. Klivenyi, N. Y. Calingasan, H. Lee, H. Kawamata, K. Loonam, A. L. Perrier, J. Bruses, M. E. Rubio, N. Topf, V. Tabar, N. L. Harrison, M. F. Beal, M. A. S. Moore, and L. Studer, "Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice," *Nat. Biotechnol.*, vol. 21, no. 10, pp. 1200–1207, 2003.

[65] Y. Shi and J. Massagué, "Mechanisms of TGF-beta signaling from cell membrane to the nucleus.," *Cell*, vol. 113, no. 6, pp. 685–700, 2003.

[66] A. Liu and L. A. Niswander, "Bone morphogenetic protein signalling and vertebrate nervous system development," *Nat. Rev. Neurosci.*, vol. 6, no. 12, pp. 945–954, 2005.

[67] C. D. Stern, "Neural induction: 10 years on since the 'default model'.," *Curr. Opin. Cell Biol.*, vol. 18, no. 6, pp. 692–7, Dec. 2006.

[68] S. M. Chambers, C. A. Fasano, E. P. Papapetrou, M. Tomishima, M. Sadelain, and L. Studer, "Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling," vol. 27, no. 3, pp. 275–281, 2009.

[69] P. Koch, T. Opitz, J. a Steinbeck, J. Ladewig, and O. Brüstle, "A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 9, pp. 3225–3230, 2009.

[70] A. Falk, P. Koch, J. Kesavan, Y. Takashima, J. Ladewig, M. Alexander, O. Wiskow, J. Tailor, M. Trotter, S. Pollard, A. Smith, and O. Brüstle, "Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons.," *PLoS One*, vol. 7, no. 1, p. e29597, Jan. 2012.

[71] Y. Elkabetz, G. Panagiotakos, G. Al Shamy, N. D. Socci, V. Tabar, and L. Studer, "Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage.," *Genes Dev.*, vol. 22, no. 2, pp. 152–65, Jan. 2008.

[72] D. Zhang, M. Pekkanen-Mattila, M. Shahsavani, A. Falk, A. I. Teixeira, and A. Herland, "A 3D Alzheimer's disease culture model and the induction of P21-activated kinase mediated sensing in iPSC derived neurons," *Biomaterials*, vol. 35, no. 5, pp. 1420–1428, 2014.

[73] J. C. Villaescusa, B. Li, E. M. Toledo, P. Rivetti di Val Cervo, S. Yang, S. R. Stott, K. Kaiser, S. Islam, D. Gyllborg, R. Laguna-Goya, M. Landreh, P. Lönnerberg, A. Falk, T. Bergman, R. A. Barker, S. Linnarsson, L. Selleri, and E. Arenas, "A PBX1 transcriptional network controls dopaminergic neuron development and is impaired in Parkinson's disease," *EMBO J.*, vol. 35,

no. 18, pp. 1963–1978, 2016.

[74] J. Tailor, R. Kittappa, K. Leto, M. Gates, M. Borel, O. Paulsen, S. Spitzer, R. T. Karadottir, F. Rossi, A. Falk, and A. Smith, "Stem cells expanded from the human embryonic hindbrain stably retain regional specification and high neurogenic potency.," *J. Neurosci.*, vol. 33, no. 30, pp. 12407–22, Jul. 2013.

[75] P. Koch, P. Breuer, M. Peitz, J. Jungverdorben, J. Kesavan, D. Poppe, J. Doerr, J. Ladewig, J. Mertens, T. Tüting, P. Hoffmann, T. Klockgether, B. O. Evert, U. Wüllner, and O. Brüstle, "Excitation-induced ataxin-3 aggregation in neurons from patients with Machado-Joseph disease," *Nature*, vol. 480, no. 7378, pp. 543–546, 2011.

[76] R. Qasim Ali, E. Blomberg, A. Falk, L. Ährlund-Richter, and M. Ulfendahl, "Induction of sensory neurons from neuroepithelial stem cells by the ISX9 small molecule," *Am J Stem Cells*, vol. 5, no. 1, pp. 19–28, 2016.

[77] M. Raciti, J. Ong, L. Weis, K. Edoff, C. Battagli, A. Falk, and S. Ceccatelli, "Glucocorticoids alter neuronal differentiation of human neuroepithelial-like cells by inducing long-lasting changes in the reactive oxygen species balance," *Neuropharmacology*, vol. 107, pp. 422–431, 2016.

[78] D. McLaren, T. Gorba, A. Marguerie De Rotrou, G. Pillai, C. Chappell, A. Stacey, S. Lingard, A. Falk, A. Smith, P. Koch, O. Brüstle, R. Vickers, J. Tinsley, D. Flanders, P. Bello, and S. Craig, "Automated large-scale culture and medium-throughput chemical screen for modulators of proliferation and viability of human induced pluripotent stem cell-derived neuroepithelial-like stem cells," *J. Biomol. Screen.*, vol. 18, no. 3, pp. 258–268, 2013.

[79] A. Lundin, L. Delsing, M. Clausen, P. Ricchiuto, J. Sanchez, A. Sabirsh, M. Ding, J. Synnergren, H. Zetterberg, G. Brolén, R. Hicks, A. Herland, and A. Falk, "Human iPS-Derived Astroglia from a Stable Neural Precursor State Show Improved Functionality Compared with Conventional Astrocytic Models," *Stem Cell Reports*, vol. 10, 2018.

[80] A. M. Palmer and S. Psychiatric, "Is the neuronal cholinergic of Alzheimer ' s disease or glutamatergic ?," *FASEB*, vol. 4, pp. 2745–2752, 1990.

[81] E. G. Gray, M. Paula-Barbosa, and A. Roher, "Alzheimer's disease: paired helical filaments and cytomembranes," *Neuropathol. Appl. Neurobiol.*, no. 1903, pp. 91–110, 1987.

[82] J.-C. Lambert, D. Mann, F. Richard, J. Tian, J. Shi, U. Thaker, S. Merrot, J. Harris, B. Frigard, T. Iwatsubo, C. Lendon, and P. Amouyel, "Is there a relation between APOE expression and brain amyloid load in Alzheimer's disease?," *J. Neurol. Neurosurg. Psychiatry*, vol. 76, no. 7, pp. 928–33, Jul. 2005.

[83] L. Bertram and R. E. Tanzi, "Thirty years of Alzheimer's disease genetics: the implications of systematic meta-analyses.," *Nat. Rev. Neurosci.*, vol. 9, no. 10, pp. 768–78, Oct. 2008.

[84] G. Talarico, P. Piscopo, M. Gasparini, E. Salati, M. Pignatelli, S. Pietracupa, L. Malvezzi-Campeggi, a Crestini, S. Boschi, G. L. Lenzi, a Confaloni, and G. Bruno, "The London APP mutation (Val717Ile) associated with early shifting abilities and behavioral changes in two Italian families with early-onset Alzheimer's disease.," *Dement. Geriatr. Cogn. Disord.*, vol. 29, no. 6, pp. 484–90, Jan. 2010.

[85] H. W. Querfurth and F. M. Laferla, “Alzheimer’s Disease,” *N. Engl. J. Med.*, pp. 329–344, 2010.

[86] J. Götz and L. M. Ittner, “Animal models of Alzheimer’s disease and frontotemporal dementia,” *Nat. Rev. Neurosci.*, vol. 9, no. 7, pp. 532–544, 2008.

[87] D. Games, D. Adams, R. Alessandrini, R. Barbour, P. Berthelette, C. Blackwell, T. Carr, J. Clemens, T. Donaldson, and F. Gillespie, “Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein.,” *Nature*, vol. 373, no. 6514. pp. 523–7, 1995.

[88] E. D. Roberson, K. Scearce-Levie, J. J. Palop, F. Yan, I. H. Cheng, T. Wu, H. Gerstein, G.-Q. Yu, and L. Mucke, “Reducing Endogenous Tau Ameliorates Amyloid b–Induced Deficits in an Alzheimer’s Disease Mouse Model,” *Biochemistry*, vol. 316, no. May, pp. 750–754, 2007.

[89] L. Duan, B. J. Bhattacharyya, A. Belmadani, L. Pan, R. J. Miller, and J. A. Kessler, “Stem cell derived basal forebrain cholinergic neurons from Alzheimer’s disease patients are more susceptible to cell death,” *Mol. Neurodegener.*, vol. 9, no. 1, pp. 1–14, 2014.

[90] C. R. Muratore, H. C. Rice, P. Srikanth, D. G. Callahan, T. Shin, L. N. P. Benjamin, D. M. Walsh, D. J. Selkoe, and T. L. Young-Pearse, “The familial alzheimer’s disease APPV717I mutation alters APP processing and Tau expression in iPSC-derived neurons,” *Hum. Mol. Genet.*, vol. 23, no. 13, pp. 3523–3536, 2014.

[91] S. H. Choi, Y. H. Kim, M. Hebisch, C. S. S. Lee, C. D’Avanzo, J. Chen, B. Hooli, C. Asselin, J. Muffat, J. B. Klee, C. Zhang, B. J. Wainger, M. Peitz, D. K. M., C. J. Woolf, S. L. Wagner, R. E. Tanzi, and D. Y. Kim1, “A three-dimensional human neural cell culture model of Alzheimer’s disease,” *Nature*, vol. 515, no. May, pp. 274–278, 2014.

[92] Y. H. Kim, S. H. Choi, C. D. Avanzo, M. Hebisch, E. Bylykbashi, K. J. Washicosky, J. B. Klee, O. Brüstle, R. E. Tanzi, and D. Y. Kim, “A 3D human neural cell culture system for modeling Alzheimer’s disease,” *Nat. Protoc.*, vol. 10, no. 7, pp. 985–1006, 2015.

[93] M. Jorfi, C. D ’avanzo, R. E. Tanzi, D. Y. Kim, and D. Irimia, “Human Neurospheroid Arrays for In Vitro Studies of Alzheimer’s Disease,” no. August 2017, pp. 1–13, 2018.

[94] J. S. Liu, “Molecular genetics of neuronal migration disorders,” *Curr. Neurol. Neurosci. Rep.*, vol. 11, no. 2, pp. 171–178, 2011.

[95] A. E. Fry, T. D. Cushion, and D. T. Pilz, “The genetics of lissencephaly.,” *Am. J. Med. Genet. C. Semin. Med. Genet.*, vol. 166C, no. 2, pp. 198–210, Jun. 2014.

[96] K. Sossey-Alaoui, a J. Hartung, R. Guerrini, D. K. Manchester, a Posar, a Puche-Mira, E. Andermann, W. B. Dobyns, and a K. Srivastava, “Human doublecortin (DCX) and the homologous gene in mouse encode a putative Ca2+-dependent signaling protein which is mutated in human X-linked neuronal migration defects.,” *Hum. Mol. Genet.*, vol. 7, no. 8, pp. 1327–32, Aug. 1998.

[97] O. Reiner, R. Carrozzo, Y. Shen, M. Wehnert, F. Faustinella, W. B. Dobyns, C. T. Caskey, and D. H. Ledbetter, “Isolation of a Miller-Dieker lissencephaly gene containing G protein beta-subunit-like repeats.,” *Nature*, vol. 364, no. 6439, pp. 717–721, 1993.

[98] M. Hattori, H. Adachi, M. Tsujimoto, H. Arai, and K. Inoue, “Miller-Dieker lissencephaly gene encodes a subunit of brain platelet- activating factor acetylhydrolase,” *Nature*, vol. 370, no. 6486, pp. 216–218, 1994.

[99] D. S. Smith, M. Niethammer, R. Ayala, Y. Zhou, M. J. Gambello, A. Wynshaw-Boris, and L. H. Tsai, “Regulation of cytoplasmic dynein behaviour and microtubule organization by mammalian Lis1.,” *Nat. Cell Biol.*, vol. 2, no. 11, pp. 767–75, Nov. 2000.

[100] G. Tian, X. H. Jaglin, D. A. Keays, F. Francis, J. Chelly, and N. J. Cowan, “Disease-associated mutations in TUBA1A result in a spectrum of defects in the tubulin folding and heterodimer assembly pathway.,” *Hum. Mol. Genet.*, vol. 19, no. 18, pp. 3599–613, Sep. 2010.

[101] H. M. Moon and A. Wynshaw-Boris, “cytoskeleton in action: lissencephaly, a neuronal migration disorders,” *WIREs Dev biol*, vol. 2, pp. 229–245, 2013.

[102] V. des Portes, J. M. Pinard, P. Billuart, M. C. Vinet, A. Koulakoff, A. Carrié, A. Gelot, E. Dupuis, J. Motte, Y. Berwald-Netter, M. Catala, A. Kahn, C. Beldjord, and J. Chelly, “A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome.,” *Cell*, vol. 92, no. 1, pp. 51–61, Jan. 1998.

[103] J. G. Gleeson, K. M. Allen, J. W. Fox, E. D. Lamperti, S. Berkovic, I. Scheffer, E. C. Cooper, W. B. Dobyns, S. R. Minnerath, M. E. Ross, and C. a Walsh, “Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein.,” *Cell*, vol. 92, no. 1, pp. 63–72, Jan. 1998.

[104] J. G. Gleeson, P. T. Lin, L. A. Flanagan, and C. A. Walsh, “Doublecortin Is a Microtubule-Associated Protein and Is Expressed Widely by Migrating Neurons,” *Neuron*, vol. 23, pp. 257–271, 1999.

[105] J. P. Brown, S. Couillard-Després, C. M. Cooper-Kuhn, J. Winkler, L. Aigner, and H. G. Kuhn, “Transient expression of doublecortin during adult neurogenesis.,” *J. Comp. Neurol.*, vol. 467, no. 1, pp. 1–10, Dec. 2003.

[106] C. a Moores, M. Perderiset, C. Kappeler, S. Kain, D. Drummond, S. J. Perkins, J. Chelly, R. Cross, A. Houdusse, and F. Francis, “Distinct roles of doublecortin modulating the microtubule cytoskeleton.,” *EMBO J.*, vol. 25, no. 19, pp. 4448–4457, 2006.

[107] L. Dehmelt and S. Halpain, “Neurite Outgrowth: A Flick of the Wrist,” *Curr. Biol.*, vol. 17, no. 15, pp. R612-14, Aug. 2007.

[108] T. Tanaka, F. F. Serneo, C. Higgins, M. J. Gambello, A. Wynshaw-Boris, and J. G. Gleeson, “Lis1 and doublecortin function with dynein to mediate coupling of the nucleus to the centrosome in neuronal migration.,” *J. Cell Biol.*, vol. 165, no. 5, pp. 709–21, Jun. 2004.

[109] T. Tanaka, F. F. Serneo, H. C. Tseng, A. B. Kulkarni, L. H. Tsai, and J. G. Gleeson, “Cdk5 Phosphorylation of Doublecortin Ser297 Regulates Its Effect on Neuronal Migration,” *Neuron*, vol. 41, no. 2, pp. 215–227, 2004.

[110] C. C. Yap, M. Vakulenko, K. Kruczek, B. Motamedi, L. Digilio, J. S. Liu, and B. Winckler, “Doublecortin (DCX) Mediates Endocytosis of Neurofascin Independently of Microtubule Binding,” *Journal of Neuroscience*, vol. 32, no. 22. pp. 7439–7453, 2012.

[111] C. C. Yap and B. Winckler, "Adapting for endocytosis: roles for endocytic sorting adaptors in directing neural development," *Front. Cell. Neurosci.*, vol. 9, no. April, pp. 1–17, 2015.

[112] J. C. Corbo, T. a Deuel, J. M. Long, P. LaPorte, E. Tsai, A. Wynshaw-Boris, and C. a Walsh, "Doublecortin is required in mice for lamination of the hippocampus but not the neocortex.," *J. Neurosci.*, vol. 22, no. 17, pp. 7548–7557, 2002.

[113] H. Koizumi, T. Tanaka, and J. G. Gleeson, "Doublecortin-like kinase functions with doublecortin to mediate fiber tract decussation and neuronal migration.," *Neuron*, vol. 49, no. 1, pp. 55–66, Jan. 2006.

[114] G. Kerjan, H. Koizumi, E. B. Han, C. M. Dubé, S. N. Djakovic, G. N. Patrick, T. Z. Baram, S. F. Heinemann, and J. G. Gleeson, "Mice lacking doublecortin and doublecortin-like kinase 2 display altered hippocampal neuronal maturation and spontaneous seizures.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 16, pp. 6766–6771, 2009.

[115] J. Bai, R. L. Ramos, J. B. Ackman, A. M. Thomas, R. V Lee, and J. J. LoTurco, "RNAi reveals doublecortin is required for radial migration in rat neocortex.," *Nat. Neurosci.*, vol. 6, no. 12, pp. 1277–1283, 2003.

[116] R. Filipovic, S. S. Kumar, and C. Fiondella, "Increasing doublecortin expression promotes migration of human embryonic stem cell-derived neurons," *Stem Cells*, vol. 30, no. 9, pp. 1852–1862, 2012.

[117] S. Cappello, C. R. J. Böhringer, M. Bergami, K. K. Conzelmann, A. Ghanem, G. S. Tomassy, P. Arlotta, M. Mainardi, M. Allegra, M. Caleo, J. van Hengel, C. Brakebusch, and M. Götz, "A Radial Glia-Specific Role of RhoA in Double Cortex Formation," *Neuron*, vol. 73, no. 5, pp. 911–924, 2012.

[118] C. Gil-Sanz, B. Landeira, C. Ramos, M. R. Costa, and U. Muller, "Proliferative Defects and Formation of a Double Cortex in Mice Lacking Mltt4 and Cdh2 in the Dorsal Telencephalon," *J. Neurosci.*, vol. 34, no. 32, pp. 10475–10487, 2014.

[119] K. Brose and M. Tessier-Lavigne, "Slit proteins: Key regulators of axon guidance, axonal branching, and cell migration," *Current Opinion in Neurobiology*, vol. 10, no. 1. pp. 95–102, 2000.

[120] H. Blockus and A. Chédotal, "Slit-Robo signaling," *Development*, vol. 143, no. 17, pp. 3037–3044, 2016.

[121] C. Fredriksson, M. Hedhammar, R. Feinstein, K. Nordling, G. Kratz, J. Johansson, F. Huss, and A. Rising, "Tissue response to subcutaneously implanted recombinant spider silk: An in vivo study," *Materials (Basel).*, vol. 2, no. 4, pp. 1908–1922, 2009.

[122] S. Rodin, A. Domogatskaya, S. Ström, E. M. Hansson, K. R. Chien, J. Inzunza, O. Hovatta, and K. Tryggvason, "Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511," *Nat. Biotechnol.*, vol. 28, no. 6, pp. 611–615, 2010.

[123] S. Rodin, L. Antonsson, C. Niaudet, O. E. Simonson, E. Salmela, E. M. Hansson, A. Domogatskaya, Z. Xiao, P. Damdimopoulou, M. Sheikhi, J. Inzunza, A. S. Nilsson, D. Baker, R. Kuiper, Y. Sun, E. Blennow, M. Nordenskjöld, K. H. Grinnemo, J. Kere, C. Betsholtz, O.

Hovatta, and K. Tryggvason, “Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment,” *Nat. Commun.*, vol. 5, pp. 1–13, 2014.

[124] M. Kele, K. Day, H. Rönnholm, J. Schuster, N. Dahl, and A. Falk, “Generation of human iPS cell line CTL07-II from human fibroblasts, under defined and xeno-free conditions,” *Stem Cell Res.*, vol. 17, no. 3, pp. 474–478, 2016.

[125] L. Zhao, Q.-L. Ma, F. Calon, M. E. Harris-White, F. Yang, G. P. Lim, T. Morihara, O. J. Ubeda, S. Ambegaokar, J. E. Hansen, R. H. Weisbart, B. Teter, S. A. Frautschy, and G. M. Cole, “Role of p21-activated kinase pathway defects in the cognitive deficits of Alzheimer disease.,” *Nat. Neurosci.*, vol. 9, no. 2, pp. 234–242, 2006.

[126] M. Nikolić, “The Pak1 kinase: An important regulator of neuronal morphology and function in the developing forebrain,” *Mol. Neurobiol.*, vol. 37, no. 2–3, pp. 187–202, 2008.

[127] A. Gdalyahu, I. Ghosh, T. Levy, T. Sapir, S. Sapoznik, Y. Fishler, D. Azoulai, and O. Reiner, “DCX, a new mediator of the JNK pathway.,” *EMBO J.*, vol. 23, no. 4, pp. 823–832, 2004.

[128] O. Reiner, A. Gdalyahu, I. Ghosh, T. Levy, S. Sapoznik, R. Nir, and T. Sapir, “DCX’s phosphorylation by not just another kinase (JNK),” *Cell Cycle*, vol. 3, no. 6, pp. 747–751, 2004.

[129] S. E. Hong, Y. Y. Shugart, D. T. Huang, S. Al Shahwan, P. E. Grant, J. O. Hourihane, N. D. T. Martin, and C. A. Walsh, “Autosomal recessive lissencephaly with cerebellar hypoplasia is associated with human RELN mutations,” *Nat. Genet.*, vol. 26, no. 1, pp. 93–96, 2000.

[130] A. G. Compton, D. E. Albrecht, J. T. Seto, S. T. Cooper, B. Ilkovski, K. J. Jones, D. Challis, D. Mowat, B. Ranscht, M. Bahlo, S. C. Froehner, and K. N. North, “Mutations in Contactin-1, a Neural Adhesion and Neuromuscular Junction Protein, Cause a Familial Form of Lethal Congenital Myopathy,” *Am. J. Hum. Genet.*, vol. 83, no. 6, pp. 714–724, 2008.

[131] N. Craddock, L. Jones, I. R. Jones, G. Kirov, E. K. Green, D. Grozeva, V. Moskvina, I. Nikolov, M. L. Hamshere, D. Vukcevic, S. Caesar, K. Gordon-Smith, C. Fraser, E. Russell, N. Norton, G. Breen, D. St Clair, D. A. Collier, A. H. Young, I. N. Ferrier, A. Farmer, P. McGuffin, P. A. Holmans, C. Wellcome Trust Case Control, P. Donnelly, M. J. Owen, and M. C. O’Donovan, “Strong genetic evidence for a selective influence of GABAA receptors on a component of the bipolar disorder phenotype,” *Mol Psychiatry*, vol. 15, no. 2, pp. 146–153, 2010.

[132] M. F. Sánchez-Font, A. Bosch-Comas, R. Gonzàlez-Duarte, and G. Marfany, “Overexpression of FABP7 in Down syndrome fetal brains is associated with PKNOX1 gene-dosage imbalance,” *Nucleic Acids Res.*, vol. 31, no. 11, pp. 2769–2777, 2003.