Engineering in Vitro Human Induced Pluripotent Stem Cell-Derived Brain Cell Models for Alzheimer's Disease and Down Syndrome

Engineering *in vitro* human induced pluripotent stem cell-derived brain cell models for Alzheimer's disease and Down syndrome

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Abstract

In disease biology, animal models are widely used. By offering insights on disease etiology, they allow us to systematically examine the complexity of a disease and its mechanisms. However, over the last decade, failures to develop therapeutic interventions resulted in substantial financial burdens on society and demonstrate that translating animal results to humans is not trivial. Although the recent stem cell technologies grant access to human substrates, it has been challenging to capture the ramifications of complicated neurological diseases within an *in vitro* two-dimensional system. Therefore, there is enormous pressure for better models for human neurological disorders, in particular, neurodegenerative and neurodevelopmental diseases. In this work, I present two case studies demonstrating the use of human induced pluripotent stem cells (iPSCs) combined with genome editing engineering and three-dimensional culture systems to recapitulate pathological phenotypes of the diseases of interest.

The first study investigates impacts of a rare variant in Alzheimer's disease (AD)-associated risk gene, ATP-binding cassette transporter subtype A7 (ABCA7), on

AD pathologies using isogenic iPSCs. Compared to the control lines derived from a healthy donor, mutant brain cells reveal a number of pathological phenotypes consistent with clinical data. Moreover, I suggest the potential contribution of malfunctioning ABCA7 to AD pathologies by showing links between the aberrant endosomal trafficking phenotypes and AD.

In the second study, I utilize three-dimensional culture systems to model Down syndrome (DS) using isogenic iPSCs derived from a DS patient. Limited accessibility to human brains and lack of appropriate in vitro model systems recapitulating the complexity of brain architecture pose a significant challenge in DS studies, particularly in developmental studies of DS. Using the cerebral organoids culture system, I observe that DS cerebral organoids can recapitulate pathological phenotypes of DS and evaluate developmental profiles compared to isogenic disomy iPSCs-derived cerebral organoids. Furthermore, I administer two classes of compounds, one for Alzheimer's disease and another for Down syndrome on DS cerebral organoids to assess the pharmacological effects on recapitulated phenotypes.

Together, these studies show that human iPSCs with genome editing and three-dimensional culture systems can serve for mechanistic and therapeutic studies on human brain diseases.

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Chapter 1: Introduction

The Necessity for a Human *in Vitro* Brain Disease Model

The brain is the central organ of the human nervous system and comprises the most complicated cellular architectures and networks. As of 2014, neurological diseases cost the United States about $ 787 billion[1]. Though substantial resources have been invested to fight various neurological diseases over decades, many remain mostly intractable. Even worse, current available therapeutic interventions for these diseases are extremely restricted despite their severity of these diseases[2–4]. This failure is partly attributed to the fact that we still do not understand brain diseases sufficiently. Many studies have relied heavily on animal models — using rats, mice, and bovines — to better understand disease progressions and mechanisms. Because they share many evolutionary similarities with ones of humans, animal models have provided invaluable, fundamental knowledge in neuroscience and tremendous opportunities to explore underlying disease mechanisms. On the other hands, animal models present several critical limitations when studying the human neurological diseases. First, it is challenging to engineer genomes in animals. The difficulties in genome editing cause challenges in discerning a plethora of genetic factors that contribute to disease progression with in vivo animal models[5]. Therefore, animal models are only suitable for modeling monogenetic diseases; they are not ideal for modeling sporadic diseases that arise from genetic mutations in multiple loci.

Second, diverging evolutionary needs causes humans to develop their own developing principles distinct from other species. The results from many studies employing animal models have shown that that non-human cell sources are not sufficient for human disease's mechanistic studies because of the human's different homology and expression levels of functional proteins, such as receptors and

transporters. Recent translational research comparing the genomic expression changes caused by insults inducing an inflammatory response in both human and mouse model highlights only 47-63% correlation range for human genes compared to the mouse orthologs, indicating a corresponding random shift in gene expression from two species[6]. Moreover, the lack of unique human cell types in animal models such as outer radial glia reinforces the interspeciesdifferences[7–9].

Third, a dramatic increase in financial burdens on therapeutic developments caused by limitations of *in vivo* studies also imposes another big challenge on the conquest of diseases. Since the clinical implications for clinical trials heavily rely on animal studies, animal models having limitations as mentioned earlier fail to recapitulate vital pathophysiological features of the human disease, resulting in poor clinical translation despite the immense financial investments.

Another obstacle in brain disease studies stems from a different angle: own characteristics of a brain itself. First, the brain is the most complicated organ system with diverse functions. It contains 10 to 100 billion neurons, resulting in 100 trillion synaptic networks[10]. Also, a brain is regionally compartmentalized for its purposes but highly interconnected for its execution. Therefore, post-mortem brain tissues as an alternative for the studies only grant a cellular and molecular snapshot of detectable alterations, failing in providing temporal and mechanistic insights into pathogenesis.

Second is immense difficulty in the access to the brain samples as well as ethical concern over their utilization. A brain is developed through organogenesis, a process to progressively build the tissues and organs of the body. It is tightly regulated by many governing principles for its integration during the development in the embryo. Since it is mostly formed *in utero*, there are immense ethical considerations, limiting access to

human brains. Furthermore, a brain has very restricted capabilities to regenerate, compared to any other organs. The limited regenerative capabilities have further restricted possible experimental platforms. Hence, our understanding in brain functions and development remains very primitive. Altogether, these limitations of *in vivo* animal models and limited knowledge of diseases have pointed toward the need for a new model that more closely reflects human biology and that serves as a platform for therapeutic approaches.

To address this need, I have worked to establish *in vitro* systems using induced pluripotent stem cells in two different disease states by combining several recent transformative technologies. In this chapter, I will introduce technologies I utilize and pitfalls of individual ones, and discuss the hurdles many scientists can encounter in disease modeling, and provide my thoughts on strategies to overcome them.

The promises of new technologies for human disease models

Pitfalls in animal studies, ethical issues over human substrates, and limited accessibility to the human brain seem quite impossible to overcome. The recent discoveries of three technologies, however, open up a new era to address those issues and to advance our biological and pathological knowledge.

In 2007, a Nobel prize-winning study of induced Pluripotent Stem Cells (iPSCs) by Yamanaka group revolutionized the way to study a brain, suggesting potential solutions to unsolved issues[11]. This reverse-engineering technique reprogramming adult somatic cells into pluripotent stem cells via retroviral infection not only provide unlimited accessibility of the human hard-to-regenerating resources but also alleviate ethical restraints in embryonic stem cells (ESCs). Furthermore, technical advances in

stem cell biology enable us to re-direct iPSCs into any cell types found in the body. In particular, with numerous methods for neuronal differentiation, it is possible to generate a sufficient number of different types of neurons in a dish for investigating disease progression, which was extremely challenging due to its limited regenerativecapability[12].

The second transformative discovery is the adaptive immune system, called clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated (Cas) genes. This system was discovered from studies on the adaptive immune system of bacteria and archaea[13]. Prokaryotes utilize this CRISPR system to defend themselves against invading foreign nucleic acids. Successful adaptation of this system to a genome-editing tool for human genome creates vast opportunities to test genetic underpinnings of many diseases by providing an easy and precise editing technology for modulating genome. Due to its simplicity and inexpensiveness, it promises great futures for the gene therapy field beyond genetic analysis[13].

Third are three-dimensional self-organizing structures, referred to cerebral organoids derived from iPSCs with specific biochemical factors. Lancaster et al. highlighted that these cerebral organoids mimic some critical aspects of the cellular composition and activity of the brain, partially reflecting the in vivo environment of a brain[14,15]. This method opens up a very likely chance to study the early developmental processes of the human brain, which used to be not achievable with conventional methods.

In chapter 2 and 3, I will show my examples to model two different *in vitro* human disease models combining technologies mentioned above, demonstrating the potentials of *in vitro* human iPSC-derived models.

Where Genetic Variability Comes From

Reprogramming methods generating human iPSCs with defined factors open up new ways to study diseases that arise from multiple genetic factors. In particular, it grants us the unprecedented access to the disease-relevant cell types carrying the precise constellation of genetic variants that caused pathological conditions in a given individual. With postmortem tissues, it was veritably impossible to discern any significant events occurred before disease onset from ones after[16]. Despite the high utilization of this technology, however, there are would-be pitfalls to which our close attention needs to be paid to draw accurate conclusions.

First, there is a possibility that each clone of human iPSC lines produced from the same individual with the same manner, can even have different properties from each other. Previous studies highlight that inherent properties of each generated pluripotent stem cell-line are affected mainly by methodological and environmental factors, such as timing and methods of derivation, and the set of culture media[17–19]. Historically, the quality examinations on generated iPSCs were executed only in qualitative manners such pluripotency immunostaining with antibodies for pluripotency, or by the teratoma formation test, one examining the transforming capability of injected iPSCs into the benign teratoma in nudemouse[20,21].

Second, different genetic backgrounds of iPSCs derived from different individuals can pose serious issues to draw precise conclusions from mechanistic experiments. Transcriptional profiling analysis from a broad set of human embryonic stem cells (hESCs) containing many cohorts of sibling pairs demonstrated that sibling lines were hierarchically clustered together regardless of deriving methods, indicating pronounced effects of genetic background over environmental effects[17]. It also has been

shown that various phenotypes of differentiated cells from different cell lines can result from variations between given iPSC lines due to the mitotic inheritability of DNA methylation[19,22]. For example, it has been demonstrated that specific cell lines are more difficult to differentiate into neural progenitors and neurons, raising risk for erroneous interpretations of phenotypes in disease modeling experiments[22,23]. Moreover, many studies provide pieces of evidence that significantly less transcriptional and DNA methylation variation between cell lines are found in male pluripotent cells compared to female ones due to increased X-linked gene expression led by a progressive. Although a series of screening process may alleviate variation risks, however, I think other extra careful considerations required for the quality control because it is laborious, but not impossible to eliminate cells in which unexpected impacts caused by a given genetic variant mask critical pathophysiological changes, depending on types of disease and its onset timing.

Overcoming the Variability Arising from induced Pluripotent Stem Cell Technology

Several approaches have been utilized to minimize risks result from the variations described above. First, while the qualitative assays such as immunostaining and teratoma formation test can evaluate only one feature, iPSCs' pluripotency, quantitative quality controlling assays such as genome-wide transcription analysis or epigenomic analysis are much beneficial to determine whether a generated line is suitable for a particularapplication[22,24]. Consequently, it must be recognized that these inherent differences between cell lines could obscure interpretations obtained from the comparison of any cell lines in the context of disease modeling experiments. Also, any

cell lines are carefully selected, and their quality controls should accompany for any given study.

Second, although large cohorts studies including both patient and healthy individuals are informative, utilizing isogenic cell lines would be an excellent way to minimize genetic variation in disease studies with multiple clones. This strategy can be compelling in such studies modeling idiopathic or sporadic forms of the disease. With the help of workable genome editing tools such as CRISPR/Cas, isogenic cell lines derived from the same individual can be generated without much difficulties and time consumption.

Lastly, thorough follow-up evaluating potential discordance via quantitative behavioral assays and transcriptional/epigenetic analysis can surmount variation issues. Interestingly, one group suggested that a quick and easy screening method, called "lineage scorecard" is efficient enough to screen reproducible and quantitative representative cell lines by a few key genes' transcription levels followed by unconstrained and random differentiating steps[24].

Handling Unanticipated Off-Target Effects in CRISPR/Cas Technology

CRISPR/Cas system is a genome-editing tool creating a double strand break (DSB) by site-specific nucleases. Despite its precise genetic editing capability at the single-nucleotide level and its many easy-to-work protocols, there is a significant concern surrounding this nuclease-based editing approach: "off-target" effects. Nuclease can induce collateral and unanticipated mutations elsewhere ("off-target") besides the target site, although several studies showed that such "off-target" mutations were found at a very low frequency[25,26]. However, Exome sequencing or the whole genome

sequencing needs to be applied to evaluate "off-target" nuclease activities. Furthermore, another concern on this technology rises from a potential risk derived from clonal selection after mutagenesis[26]. Since the clonal isolation step itself impose risks for unexpected mutations that lead to pathological phenotypes, masking effects of the intended modification, multiple clones followed by experimental validation of any subsequent phenotypes observed would be necessary. Moreover, before creating an isogenic line, the parental cell line needs to be assured that it does not show any atypical pathological phenotypes and have differentiating capability into different cell types of interest.

Needs for Three Dimensional Models With More Physiologically-Close Conditions

Self-patterned cerebral organoids can provide unprecedented opportunity to study healthy brain development and disease. This reductionist's approach for developing human brain enables us to access brain tissue recapitulating cellular diversity and circuit functionality. Thus far, this system has been applied to study neural progenitor dysfunction cases during early brain development stages, includingmicrocephaly[15], Zika virus infections[27,28], and autism spectrum disorder[29].

Another strategy to build three-dimensional culture system is to use biomaterials. Employing the biomaterials such as hydrogels enables researchers to engineer three-dimensional models mimicking tissue-specific architectures or pathological phenotypes that have not shown in two-dimensional *in vitro* models[30,31].

When iPSC technology meets genome-editing technology

The combined approach using two transformative technologies can further

enhance our understanding of disease biology by providing a better platform. Combining these technologies, two possible manners in modeling diseases are selectable: one is to add (a) risk allele(s) to the undiagnosed "normal" background, and another is to remove one or multiple suspected mutation(s) from pathological backgrounds. In the recent era, plentiful risk variants for many different disease states are identified by large-scale of Genome-wide association studies (GWAS)[32–34]. First approach using "normal" background can directly test causative effects of an inserted variant identified from GWAS in a target disease. On the other hand, in many cases, risk variants uncovered by GWAS are the common allele, which has a little contribution to the disease progression but higher penetration. Thus desirable pathological phenotypes cannot be manifested with this approach if inserted variant had minimal impacts on pathological conditions. Subtracting approach removing a variant of interest can also test its causative effects on the conditions. In particular, it can grant an interesting opportunity to explore unknown disease-causing mutations in neurological disorders with a strong genetic basis[35]. Furthermore, one advantage of the latter approach over the first is to require no consideration of a potential risk of "protective background". In a case that a parental cell line already has this protective genetic background, introducing a risk variant into a genome cannot invoke any pathological manifestation. On the other hand, using a patient-derived iPSCs approach, this protective genetic background cannot confound effects of a variant in question. One possible downfall in this approach is the availability of specific patient-derived iPSCs that have the "right" genetic background harboring a variant in question. Though both approaches can provide invaluable information, to determine which is a more suitable approach, such thorough consideration about a type of disease and available information on risk factors needs to

be pondered.

Selecting Differentiating Protocols

Once establishing iPSC lines for disease modeling with necessary validations, next step is to determine how to differentiate iPSCs into cell types of interest. Though many different protocols for various neuronal cell types and different glia cell types have beenreported[36–38], those protocols need to be carefully reviewed and evaluated, based on the experimental purposes and needs such as the ability to generate enough cell numbers, specificity of differentiated cell type, and heterogeneity of produced cell types.

The productivity of cell type to interest is critical criteria in selecting protocols. While there are such experiments as immunocytohistogram and electrophysiology requiring a relatively small number of cells, various examinations are demanding a large number of cells for analysis. For example, RNA-sequencing experiments or the "-omics" type of assays require millions of cells[39].

Next, once differentiating protocols are set up, the presences of cell type in question can be confirmed in various ways: by immunocytochemistry, by transcriptional profiling, and by functional assays. Although the immunocytochemistry method is often used for the rapid evaluation, it is not uncommon that gene expression signatures identifying cell type to interest are not available. Then functional assays can be great alternatives. For instances, spinal motor neurons can be validated through neuromuscular junction formation[40] whereas neurosecretory cells can be verified by peptide secretion[41].

Previous studies demonstrated that different cell types in the brain contribute to the disease progression in a distinct manner[42,43]. Therefore, a conclusion drawn from cultures containing diverse cell types as a whole can be misled. Setting up another step

of purification can diminish unintentional effects stemming from cellular heterogeneity via fluorescent activated cell sorting (FACS) or magnetic separation using appropriate surface markers[44,45].

Conclusion

iPSCs-based disease models are promising *in vitro* platforms for mechanistic studies and drug discovery for diseases. Notably, it grants us the unprecedented access to the human substrates, which can compensate critical disadvantages of animal models failing to recapitulate human phenotypes. On the other hand, iPSC-based technologies also pose several inherent challenges to be addressed in *in vitro* disease modeling. Thus, careful experimental designs in which, if any, other suitable technologies can be applied should be considered for a better *in vitro* disease modeling system.

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Chapter 2: Developing Human Induced Pluripotent Stem Cell (iPSC)-Derived Brain Cell Type-Specific Disease Model of Alzheimer's Disease (AD)

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease, resulting in severe memory loss. Late-onset sporadic AD (sAD) is a subset of AD defined by its onset age and unspecified genetic cause. Although neuronal nature of AD is widely accepted, there is increasing recognition of the role of non-neuronal cells in the disease phenotype. While sAD can be attributable to many genetic causes, the evidence is evolving on previously unrecognized genetic etiologies from the recent genome-wide association studies (GWAS). Thus, there is an urgent need to better understand the genetic contribution to the pathophysiology of sAD, which will help to inform the course of disease progression and treatment options. ATP-binding cassette transporter subtype A7 (ABCA7) belongs to one of subfamily A type of ABC transporters that function to regulate the homeostasis of various lipids in the central nervous system, but very little is known regarding ABCA7's function in AD. I have derived induced pluripotent stem cell (iPSC) from a healthy individual and created isogenic mutant knock-in harboring ABCA7-Y622* via CRISPR/Cas9 and have shown that this *in vitro* model mimics three key features of the disease: (1) elevated amyloid β (Aβ) production; (2) impaired Aβ uptake capability; (3) aberrant endosomal phenotype. While Aβ production and up-take are specified to corresponding cell types, abnormal endosomal trafficking is prevalent through all brain cell types. Furthermore, I observed that lipid compositional changes are induced by the ABCA7 mutation, which possibly leads to endosomal trafficking impairments. Together, my data highlight the utility of iPSCs in providing a disease model of AD and implying a potential link of a genetic risk factor to the

pathophysiology of AD.

Introduction

Alzheimer's Disease (AD) is a progressive, neurodegenerative disease featured with severe memory loss[56]. It affects a wide range of the cerebral cortex and hippocampus, starting with the frontal and temporal lobes, and later progressively neocortical regions. There are about two decades of preclinical and prodromal stages before the clinical symptomatic phases, which last about 8-10 years. Pathological symptoms of AD are associated with accumulated insoluble amyloid-β (Aβ), called plaques, and aggregation of the microtubule protein tau as a form of neurofibrillary tangles in neurons[1]. Amyloid-β peptides, the main constituent of senile plaques, are produced in neurons by the proteolytic cleavage of the protein, called amyloid precursor protein (APP) through a series of enzymatic activities including β-secretase and γ-secretase.

There are two types of Alzheimer's disease: late-onset, sporadic AD (sAD) and early-onset, autosomal dominant inherited form, called familial AD (fAD). Over 95 percent of all Alzheimer's disease cases are sporadic Alzheimer's disease[2] and its average onset age is 80 years although causing factors are largely diverse. Despite the high prevalence, clear genetic associations of sAD have been not identified. On the other hand, familial AD (fAD) representing less than 5 percent of the entire cases, has the mean onset age of 45 years, and mutations on genes, such as APP, Presenilin 1 (PS1), and Presenilin 2 (PS2) are known to cause familiar AD (fAD)[3]. All three causal genes, APP, PS1 and PS2 of fAD are associated with overproduction or formation of a pathological and aberrant form of Aβ.

Despite the fact that the overall burden of cognitive impairment in the elders

is estimated through the many epidemiological studies on dementia, the common comorbidities at the age of 80 years, such as cerebrovascular disease and hippocampal sclerosis, complicate and mislead diagnosis and management of Alzheimer's disease. New diagnostic technologies for detecting AD-specific markers such as tau and Aβ and for molecular PET imaging in living patients are now becoming available, which not only reliably track pathological changes over brains, but also enhance to precisely evaluate the prevalence and incidence of Alzheimer's disease. They also help to develop primary and secondary preventive interventions for Alzheimer's disease.

Investigating mechanisms of Alzheimer's disease can provide new insights into the pathogenesis, diagnosis, and treatment of the disease. However, it is not easy to evaluate putative associations with genetic risk factors due to elements, such as confounding variables of comorbidities and diversity in genetic background. Therefore, in spite of lower incidence, Alzheimer's disease studies have long been focused on fAD cases due to the Aβ peptides and its causative mutations, such as PS1 and PS2 and interrogated with a neuron-centric view. However, recent large-scale genetic studies have identified numerous risk genes primarily expressed in non-neuronal cell types, such as astrocyte and microglia as significant risk factors for sporadic Alzheimer's disease, suggesting the importance in regulating the balance between production and clearance of this peptide from the brain[4,5].

ATP-binding cassette transporter subtype A7 (ABCA7) was identified as an AD-associated gene by recent genome-wide association studies (GWAS)[6]. ABCA7 is a member of the A subfamily of ABC transporters, being highly expressed in microglia

in the brain[7]. While much effort has made on understanding the mechanisms of AD driven by fAD mutations, specific impacts of ABCA7 on sAD, however, remain much less clear. Most studies on the effect of ABCA7 on sAD have relied on knockout mouse model and non-brain cell lines, such as HEKs[8,9]. ABCA7 knockout mouse studies have shown that cerebral amyloid β plaque load was exacerbated[8]. Besides, results from *In vitro* cellular models suggest that ABCA7 may affect phagocytic activities[10,11], but the role of ABCA7 in phagocytosis is not entirely clear. Despite these studies, the cell type-specific effects leading to pathological phenotypes have not directly tested. Moreover, studies utilizing animal models have drawn a lot of attention and concerns about translatability to humans due to species differences[12]. Similarly, current *in vitro* studies have revealed limitations caused by inaccessibility to the relevant cell types such as neurons and glial cells and by failure to build complex disease model.

Here, in order to address the aforementioned issues and bridge a gap in our understanding of ABCA7 effects on AD pathogenesis, I have combined reprogramming stem cell differentiation approaches with genome engineering technologies, called Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/ CRISPR-associated gene 9 (Cas9) to identify functional changes induced by the ABCA7 mutation in human brain cells. For this study, isogenic iPSC lines harboring homozygous rare mutant (Y622*) alleles from healthy parental control cells. A rare variant was selected instead of common variant because the rare one presents stronger association with sAD in contrast to the common variant that has the weak association with AD. My study using isogenic hiPSCs carrying the ABCA7

variant highlights that harboring this variant is sufficient to recapitulate pathological hallmarks of AD at multiple levels within brain cells, such as iPSC-derived neurons, astrocytes, and microglia-like cells. Furthermore, the results from functional assays also exhibit several ABCA7-dependent phenotypes with the potential to induce pathological conditions in AD patients: ABCA7 knock-in neurons revealed increased Aβ42 secretion compared to the control, while both ABCA7 knock-in astrocyte and microglia-like cells exhibited impaired Aβ uptake and functions in clearance. In addition, ABCA7 knock-in 3D *in vitro* models displayed elevated level in amyloid accumulation and tau phosphorylation relative to the control, which is consistent with the late-onset, sporadic AD pathology. Notably, I found that mutant ABCA7 impairs a delicate balance of endosomal trafficking system in all the cell types. My study suggests unprecedented insights into the multiple roles of ABCA7 in AD development and provides a platform for mechanistic studies on ABCA7-dependent pathogenesis as well as insights into therapeutic interventions for AD.

Results

Generating human isogenic ABCA7 Knock-In (K.I) iPSCs

In order to assess the effects of the ABCA7 variant on specific brain cell types, I used CRISPR/Cas9 genome editing technologies to generate isogenic ABCA7 iPSC lines from ABCA7 control cells, which are derived from a healthy individual with no diagnosis of Alzheimer's disease (Figure 2.1A). As a first step, the Cas9 protein, a single guide RNA (sgRNA) was inserted to ABCA7 control cells, targeting the relevant region of ABCA7 based on the selected rare variant with the strongest odd

ratio[13], and a repair template, single-stranded oligodeoxynucleotides (ssODNs) was introduced to direct precise edits of ABCA7 (Figure 2.1B). In the following step, DNA extraction procedure was executed from a single colony made out of a single cell after the mutagenesis, and its Sanger sequencing was performed to measure the success of editing of the ABCA7 locus by checking a point mutation corresponding to the Tyrosine-622-stop substitution (ABCA7 KI) (Figure 2.1C).

Neuronal differentiation of isogenic ABCA7 iPSCs

Neurons are the major cell type having the most ABCA7 expression[14]. Thus, I derived isogenic ABCA7 iPSCs into neurons through introducing Neurogenin-2 (Ngn2) into the genome. I followed the protocol created from the Südhof lab[15] with slight modifications. Instead of co-culturing with astrocytes, human astrocyte-conditioned media was used in order to increase the purity of neuronal populations. Immunostaining results show that after four weeks induction, both isogenic ABCA7 neurons derived from iPSCs strongly expressed neuronal markers, such as MAP2 as well as the postsynaptic markers, such as PSD95, not neural progenitor markers, Nestin (Figure 2.2A). I also performed the electrophysiology on both lines and observed fired action potentials were from both lines, indicating their maturity (Figure 2.2B).

sAβ_{42} and sAβ_{40} secretion in ABCA7 neurons

Neurons have long been considered as a central player in the brain disease. In particular, for Alzheimer's disease, neurons are not only the major contributor to

the AD pathologies as the primary Aβ producers but also the most susceptible cell type to degenerative damages in the AD conditions. Increased levels of Aβ production has been the most studied characteristic of AD pathologies[16,17]. To determine whether ABCA7 can affect aberrant Aβ release, I measured the Aβ secretion levels from neurons by ELISA. I found that ABCA7 KI has much higher levels of $A\beta_{40}$ secretion compared to the ABCA7 control cultures. Furthermore, the higher levels of $A\beta_{42}$ secretion was also detected in ABCA7 K.I neurons whereas no $A\beta_{42}$ was observed in the control neurons. As a result, the $A\beta_{42}$: $A\beta_{40}$ ratio was increased in ABCA7 K.I neuron culture media (Figure 2.2C). To determine whether this elevated Aβ production extended to other neuronal deriving culturing systems, and whether these systems can recapitulate Aβ aggregates that plain two-dimensional *in vitro* system cannot mimic, I utilized two three-dimensional culturing systems: one is a scaffold-based culture system, and another is a self-assembly based system, called cerebral organoids (Figure 2.2A). In a scaffold-based system, I observed that accumulated Aβ aggregations were formed only in the three-dimensional culture containing ABCA7 K.I neurons, not in the control cultures (Figure 2.2D). In addition, increased levels of both Aβ40 and Aβ42 were detected in cerebral organoids derived from ABCA7 K.I iPSCs, compared to ones from the control iPSCs (data not shown). In the immunoblotting analysis, I observed that APP expression level was slightly elevated in ABCA7 K.I organoids compared to the control ones (Figure 2.2D).

<u>Abnormal endosomal phenotypes in ABCA7 neurons</u>

Previous studies describe that elevated Aβ production can be led by several factors, such as abnormal endosomal activities: increased cleaving activities of β-site APP-cleaving enzyme 1 (BACE1) on amyloid precursor protein (APP), and the β c-terminus fragment (β-CTF), a precursor of Aβ[18]. Moreover, aberrant early endosomes in number and size has long been reported in AD patients' brain[19]. Thus I examined whether ABCA7 K.I can induce detectable endosomal changes in neurons by immunostaining with an antibody against early endosome antigen 1 (EEA1), a marker for early endosome. The results show that ABCA7 K.I neurons have increased number of large size of EEA1+ particles (>1μm^2) compared to the control, whereas the number of smaller size (<1μm^2) of endosome counterparts was indistinguishable (Figure 2.2E). Together, iPSC-derived neurons harboring the ABCA7 variant exhibited such strong known AD phenotypes, such as elevated Aβ secretion, Aβ aggregation, and enlarged early endosomes, suggesting their crucial roles in sAD etiology in patients carrying this risk allele with its high odds ratio.

Generation of isogenic ABCA7 astrocytes

Astrocytes, a major type of glial cell, are well documented to play essential roles in neurodegenerative disease as well as regulating acts for the synaptic formation of neurons[20,21]. However, the effects of ABCA7 on astrocyte function remain largely unknown. For generating astrocytes, I performed two-step differentiating processes: first making neural progenitor cells (NPCs) by two growth factors, SM431542 and Dorsomorphin, then deriving NPCs to astrocytes with an additional growth factor, BMP-4, following the protocol from the Deng group[22]

(Figure 2.3A). The derived cell type was confirmed by the immunostaining with the antibody against S100b, astrocyte marker (Figure 2.3A).

Aβ clearing capacity

Astrocytes are known to provide neuroprotective functions in AD by clearing oligomeric/fibrillar $A\beta_{42}$ *in vitro* and *in vivo*, which may result in preventing detrimental effects of amyloid accumulation[23–25]. Thus, I determined whether the ABCA7 variant affected the ability to internalize Aβ. In order to address this question, I measured the Aβ clearance ability of my isogenic astrocytes. Considering relatively slow internalization rate of astrocytes, I administered known concentration of synthetic $A\beta_{42}$ oligomers for two days and measured the $A\beta_{42}$ level left over by ELISA. For the quantification, I used an uptake index to estimate the fraction of oligomeric $A\beta_{42}$ removed from the media including the self-degrading amount. Furthermore, I also confirmed to measure no detectable amount of Aβ secreted from astrocytes, confirming that there is no endogenous contribution of Aβ from astrocytes. My data show that ABCA7 K.I has less efficient than the control in internalizing Aβ42 from the media (Figure 2.3B).

Although aberrant endosomal size and number of neurons were reported in AD[19], it has not been studied that astrocytes also have the same phenotypes. Thus, performing the immunostaining with an antibody against early endosome, I examined the size and number of early endosomal puncta and confirmed that ABCA7 K.I astrocytes have enlarged and increased number of early endosomes compared to the control, which was consistent with the results in neurons (Figure

2.3C). Taken together, astrocytes harboring the ABCA7 K.I variant impair Aβ42 uptake and endosomal trafficking, which both could contribute to AD pathologies.

Induction of isogenic iPSCs into Microglia-like cells (MGLs) and A clearance

Microglia are another glia-type cells residing in the brain, playing significant roles in modulating inflammation and having phagocytic functions in the central nervous system (CNS)[26]. A number of recent genetic studies highlight that microglia have various roles in brain health and that their dysfunctional activities can lead to sAD progression[27–29]. Nevertheless, despite the high expression level of ABCA7 in microglia, its effects on microglia remain largely unknown. To evaluate the impact of ABCA7 on microglia, I used the published protocol from the Jaenisch group to differentiate toward microglia-like cells[30] and then performed immunostaining with an antibody against ionized calcium-binding adaptor molecule 1 (Iba1), microglia marker (Figure 2.4A). Next. In order to explore ABCA7 impacts on microglia's phagocytic activities, I examined Aβ internalizing capabilities and found that ABCA7 K.I microglia have less internalized Aβ compared to the control microglia (Figure 2.4B). To rule out the possibility that the amount of Aβ decreases by faster turn-over of Aβ of microglia, I performed live-imaging of $A\beta_{42}$ uptake of microglia for an hour using fluoresce-conjugated $A\beta_{42}$ ($A\beta_{42}$-555). During the period of one hour, ABCA7 microglia-lie cells internalized $A\beta_{42}$ much slowly and less than the control did (Figure 2.5A). Interestingly, I noticed that less $A\beta_{42}$ were bound to the membrane of ABCA7 K.I microglia (Figure 2.5A).

I also interrogated microglia's endocytic activities using an endosomal

marker, EEA1 to determine whether this modular process was also affected by ABCA7 similar to other cell types, and found that ABCA7 K.I microglia have more enlarged early endosome particles compared to the control (Figure 2.4C).

Abnormal endocytosis and endocytic trafficking jam

Endocytosis is an endocytic portal into cells through which cargo is packaged into vesicles with the help of various coating proteins. Vesicles formation is continuously progressed throughout multiple stages via various protein complexes. Thus, disruptions in any step can cause aberrant transports. To examine whether any trafficking disruption induced deficits in Aβ uptake derived from ABCA7 K.I, I traced lysosomal activities, in where degradation of macromolecules occurs. First, in order to measure the basal lysosomal activities, I administered fluorescence-tagged lysosomal tracer (lysoTracker™) to microglia-like cells due to its fast rate of endocytosis so that its events can be traced in real time. As predicted, lysotracker in ABCA7 K.I microglia had been degraded much slower, indicating down-regulated baseline of lysosomal activities (Figure 2.5B). Then I introduced oligomerized Aβ42 with lysotracker into MGLs to test if the AD pathological conditions could aggravate lysosomal activities. The data highlight that lysosome got enlarged and remained active much longer than the controls, indicating deficits in degradation (figure 2.5C). To further test whether degradation deteriorates, I examined the co-localizing signals of oligomerized Aβ42 and lysosome and found that ABCA7 K.I microglia-like cells have more intensified co-localizing signals and larger volumes of compartments containing both Aβ42 and lysotracker, further

supporting less degradation in ABCA7 K.I lines (Figure 2.5D).

Lastly, in order to determine whether this abnormal endocytic phenotype is specific only to Aβ peptide, I used transferrin proteins, peptides that are internalized via clatherin-mediated endocytosis. Our data highlighted the enlarged trafferin aggregates, indicating overall defects in endocytic trafficking systems (Figure 2.5E).

Lipids and endosomal trafficking

Despite little knowledge of ABCA7's roles, because of the structural similarities with ABCA1, which is the most studied lipid transporter[31], ABCA7 be considered as a tentative lipid transporter as well. Lipids are one of the molecules actively transported between various compartments such as membrane and ER. They also regulate endosomal trafficking systems by modulating the delicate balance between compartments[32,33]. Therefore, any imbalance in lipid composition may affect endocytic trafficking systems. To examine whether the mutation induced any compositional changes in lipids on the membrane, I performed the immunostaining with different antibodies against different kinds of lipids. I observed many changes in many lipid types that can modulate endocytic pathways directly or indirectly (Figure 2.6A). I also tested other membrane-bound proteins which distribution is actively regulated by endocytic trafficking systems and observed its distribution pattern and signals were affected by the ABCA7 K.I variant in microglia-like cells (Figure 2.6B). Cellular morphology is another area where cells dynamically modulate lipid compositions through active transporting systems for various behavioral purposes[34,35]. Our data highlight that ABCA7 K.I microglia have

shorter and fewer processes when treated with oligomerized Aβ42, suggesting limited capability in mobility and detection (Figure 2.6C). Together, microglia-like cells harboring ABCA7 K.I mutation have deficits in endosomal and lysosomal activities. Besides, distinct patterns and reduced levels of various lipid compositions were observed in ABCA7 K.I lines compared to the control.

Discussions

To evaluate the impacts of rare ABCA7 variants on AD-related phenotypes in each brain cell type, I utilized the genome-editing tool on iPSCs derived from a healthy individual and generated isogenic ABCA7 cell lines harboring the ABCA7-Y622* mutation. Surprisingly, we observed multiple cellular phenotypes related to AD pathogenesis in iPSC-derived neurons, astrocytes, and microglia-like cells. Though further investigation is needed to test whether ABCA7 can directly affect AD-related phenotypes or via other pathologies related to ABCA7, our findings suggest that ABCA7-Y622* mutation is sufficient to induce AD pathological phenotypes.

ABCA7 and Aβ production

Our iPSC-derived neuron data exhibited that secretion of soluble Aβ isoforms was elevated in ABCA7 K.I neurons, compared to the control. Consistently, three-dimensional culture systems from ABCA7 Knock-In iPSCs revealed more Aβ aggregates and increased level of pTau. These results are consistent with other in vivo studies showing that ABCA7 deficiency exacerbates Aβ plague burden[36,37]. Thus,

our evidence indicates that deficits in ABCA7 facilitate Aβ production.

ABCA7 and Aβ clearance

The genetic network studies point that genes expressing in glial cells including CR1, SPI1, CD33, TREM2, and ABCA7 are associated in sAD[27], indicating the growing role of glial cells in the disease pathogenesis. In particular, microglia heavily involves in the cellular uptake of Aβ[38]. Another study shows that microglia phagocytize Aβ, thereby regulating proteostasis of Aβ[39]. Also, several studies show that a cell's phagocytic function can be impaired by downregulated ABCA7[10,11,40]. Thus, our data revealing that ABCA7 mutation significantly impairs the capability to uptake Aβ in glial cells suggest that ABCA7 predominantly modulate Aβ clearance.

Aβ pathologies and endocytosis

In addition to the cell type-specific functional abnormalities such as neuron's Aβ production and glia's Aβ uptake, out data highlight that the increased size and number in early endosomes were found in common from all three tested brain cell types. This result is striking because aberrant endocytic trafficking can affect both Aβ production and Aβ clearance. Regarding Aβ production, endocytic trafficking plays a critical role in Aβ processing because Aβ is proteolytically cleaved from APP by a series of enzymatic activities in early endosomes[41]. In order for APP to be processed, internalization of APP is the most critical step[42]. Several studies also show that modulating the genetic expression of ABCA7 can affect the amount of Aβ generation[43,44].

While the endocytosis of APP is a significant step for Aβ production, impairment in endocytosis can clearly modulate microglial phagocytic capability because endocytosis is one type of phagocytic activities.

Therefore, our results are consistent with previous reports, supporting that ABCA7 can induce dysfunctional endocytic trafficking, thereby impairing both Aβ generation and Aβ clearance. Further study is required to determine whether ABCA7 can directly affect APP and Aβ endocytosis or indirectly modulate trafficking through other proteins.

ABCA7 and endocytosis

Many studies show that lipids can affect endocytosis in various ways: lipids can play a role in endocytosis as a mediator[45], and as a modifier[46]. In addition, It has been well known that lipid compositions on membranes of each organelle direct endocytic trafficking systems[47]. As predicted from the similar structure of ABCA1, ABCA7 play a role in lipid metabolism. One study shows that ABCA7 can alter phospholipid profile in mouse brain[37]. Also, a number of studies indicate that endocytosis of extracellular Aβ can be impaired by lipid compositions in lipoproteins[48,49]. Besides, many *in vitro* studies highlight that ABCA7 expression can modulate a cell's lipid profile[50–53].

Together, our studies suggest that ABCA7 plays a potential role in maintaining lipid compositions, thereby regulating endocytic trafficking, which can significantly contribute to AD pathologies in manners described above.

Furthermore, previous studies show that any interrupted lipid regulation evoked by

abnormal ABCA7activities can cause cell cycle arrest and that ABCA7's absence can disrupt cellular development and function[54,55]. Therefore, our data also propose that ABCA7 can contribute to AD pathologies by disrupting cellular homeostasis.

My study is the first to reveal the multifaceted effects of ABCA7 on diverse cellular and molecular phenotypes across multiple human brain cell types that collectively affect not only amyloid accumulation but also other aspects of AD pathogenesis, such as amyloid clearance. Together with the cell type-specific changes I observed, these results provide the better understanding of the role of ABCA7 in Alzheimer's disease and suggest potential therapeutic ideas for ABCA7-associated AD pathology.

Experiments Procedure

Human Induced Pluripotent Cells (hiPSCs) Cultures Human healthy iPSC line (Coriell #AG09173) was kindly shared by Bruce Yankner *laboratory*. Depending on the experimental purposes, iPSCs were cultured and maintained either on Matrigel (Corning) with mTeSR™1 media (Stem Cell Technologies) or on irradiated mouse embryonic fibroblasts(MEFs, MIT-GlobalStem) in human ES (hES) media, DMEM/F12, HEPES media (Gibco) supplemented with 20% knockout serum replacement (KSR, Gibco), 1X non-essential amino acids (NEAA), 1X GlutaMax (Life Technologies), 12 nM β-fibroblast growth factor (βFGF2, PeproTech) and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich). All iPSCs were maintained at 37 ˚C and 5% CO_2 in a humidified incubator.

ABCA7 Isogenic iPSC Lines Generation The CRISPR/Cas9-ABCA7 sgRNA

plasmid was prepared followed by the published protocol (Ran et al., 2013). Briefly summarizing, a sgRNA sequence within 10 nucleotides from the target site corresponding to amino acid 120 was designed as the CRISPR/Cas9 Design Tool (http://crispr.mit.edu) suggested. The oligomer pairs (forward: 5'-CACCGCCCCTACAGCCACCCGGGCG-3' and reverse: 5'-AAACCGCCCGGGTGGCTGTAGGGGC-3') were annealed and cloned into pSpCas9-2A-GFP (PX458) plasmid (Addgene #48138). Plasmid DNA was submitted for Sanger sequencing to confirm correct ABCA7 sgRNA sequence. Furthermore, single-strand oligooxynucleotides (ssODN) was designed to create the non-sense mutation on ABCA7: 5'-GGTGCGCGCCCCCAGGCCAATCCAGGAGCTGCACCCTAAGCTCCCGTTGCCTCTCACAGCT GGGAGACATCCTCCCCTAGAGCCACCCGGGCGTCGTCTTCCTGTTCTTGGCAGCCTTCGCG GTGGCCACGGTGACCCAGAGCTTCCTGCTCAGCGCCTTCTTCTCCCGCGCCAACCTGG-3'.

For electroporation, 80% confluent iPSCs of healthy line (#AG09173) were dissociated with 20 minutes-long accutase treatment (Thermo Fisher Scientific) supplemented with 10 μM ROCK inhibitor (Tocris). About 5 million cells were subject to electroporation using Amaxa and Human Stem Cell Nucleofector Kit I (Lonza) according to the manufacturer's instructions. In short, cells were resuspended in 100 μl of reaction buffer from the kit supplemented with 7.5 μg of CRISPR/Cas9-ABCA7 sgRNA plasmid and 15 μg of ssODN. This mixture was nucleofected with program A-23, then resuspended with hES media supplemented with 10 μM ROCK inhibitor and seeded on MEF plates. To screen the cells having successful integration of CRISPR/Cas9-ABCA7 sgRNA plasmid into host genome,

fluorescence-activated cel sorting (FACS) was performed two days after the electroporation. In brief, cells were accutased and filtered through Falcon polystyrene test tubes (Corning #352235). Filtered cell suspensions were transferred to Falcon polypropylene test tubes (Corning #352063) and sorted by BD FACS Aria IIU in FACS Facility at the Whitehead Institute. After sorting, cells were resuspended in hES media supplemented with 1X Penicillin-Streptomycin (P/S, Gemini Bio-products) and 10 μM ROCK inhibitor, and seeded on 6 wells MEF plates.

Colony inspection each colony grown out of FACS-performed single cells was transferred to one well of gelatin-coated 12-well plate covered with MEFs and maintained till the colony grew big enough for another transfer. A part of each colony was transferred to 12-well plate while the rest of iPSCs in the original plate were collected and used to extract genomic DNA. Using primers (5'-CTGGTTCTGGTGCTCAAG-3' and 5'-CCTACGGCAGACGTCTTCAG-3'), DNA in ABCA7 gene was amplified and its PCR products were submitted to GENEWIZ for Sanger sequencing.

Karyotyping In order to assess any abnormalities in chromosomes of iPSCs, we performed karyotyping after inspecting colonies. Cells cultured on Matrigel (Corning) in mTESR™1 media (Stem Cell Technologies) were prepared, and then sent to Cell Line Genetics for the analysis.

Excitatory Neuron Differentiation We followed the published protocol (Zhang et al., 2013) to generate human iPSC-derived excitatory neurons. In summary, About two millions of iPSCs cultured on Matrigel-coated 6-well plates were prepared. Lentivirus harboring rtTA and Ngn2-GFP expression vectors were mixed with

mTeSR™1 media with 2 µl Thiazovivin (Tocris) and administered to the plates (Day 0). After 24 hours (on day 1), the full media were replaced with DMEM/F12 supplemented with 1X N2 and 1X NEAA (Thermo Fisher Scientific). In order to induce TetO gene expression, following factors were added to the new media: 10 ng/ml BDNF (Peprotech), 10 ng/ml NT-3 (Peprotech), 0.2 µg/ml Laminin (Corning), and 2 µg/ml Doxycycline (Sigma-Aldrich). On day 2, another full media were removed and replaced with Neurobasal media supplemented with 1X B27 and 1X GlutaMax (Thermo Fisher Scientific) containing 10 ng/ml BDNF (Peprotech), 10 ng/ml NT-3 (Peprotech), 0.2 µg/ml Laminin (Corning), 2 µg/ml Doxycycline (Sigma-Aldrich), and 1 µg/ml Puromycin (Millipore). On Day 4 (two days after Puromycin treatment), cells were purified based on GFP expression by FACS, and re-plated onto Matrigel-coated plates. As of this step, human astrocyte (ScienCell, #1850)-conditioned media supplemented with 10 ng/ml BDNF, 10 ng/ml NT-3, 0.2 µg/ml Laminin and 0.5 Doxycycline were used for further maintenance. On day 5, 1 µM Ara-C (Sigma-Aldrich) was added to conditioned media for the purification and kept in media until day 8. On day 9, the full media were removed and replaced with human Astrocyte-conditioned media with the same concentration of BDNF, NT-3, Laminin, and Doxycycline. Only half volume of media change was performed every four days and maintained until cells were ready for experiments.

Astrocyte Differentiation 100% confluent Matrigel-grown iPSCs were prepared, cultured in mTeSR™1 media. As of this point, cells were cultured with neural media (1:1 mixture of DMEM/F12 GlutaMax, Neurobasal) supplemented with 1X N-2, 1X B-27, 1X NEAA, 1X GlutaMax, 1X P/S, 5 µg/ml insulin, 100 µM 2-

mercaptoethanol instead of mTeSRTM1 media. For induction, 1 μM Dorsomorphin and 10 μM SB431542 were added into neural media for 12 days. Then cells were accutased and passaged to new Matrigel-coated plates. Once the neural rosette structure was identified under the microscope on day 16-24, another passage was performed with four million cells/well seeding density. A day after the passage, 20 ng/ml FGF2 and 10 ng/ml Bone Morphorogenetic Protein 4(BMP4, Peprotech) were added into neural media for maintenance. Full media change was performed every other day for 28 days. Using GLAST antibody (Miltenyi Biotec), astrocytes were purified from the mixed population by FACS. After sorting, purified astrocytes were cultured in astrocyte media (Sciencell) every other day for 7 days, and were FACS-sorted again for further purification with GLAST surface marker antibody.

Microglia-like Cell Differentiation Microglia-like cells were derived from iPSCs as previously described (Muffat et al., 2016). In short, iPSCs grown on MEFs plates were prepared. Cells were dissociated by Collagenase IV (Thermo Fisher Scientific), and resuspended in MGD media (Neurobasal media supplemented with 0.5X Gem21 (Gemini Bio-products), 0.5X Neuroplex N2 (Gemini Bio-products), 0.2 Albumax I (Thermo Fisher Scientific), 5 mM Sodium Chloride (Sigma-Aldrich), 1 Sodium Pyruvate, 1X P/S, 1X GlutaMax, 3.5 ng/ml Biotin (Sigma-Aldrich), 10 μM Ascorbic acid (Sigma-Aldrich) and 1.7% Lactic syrup (Sigma-Aldrich)) with 10 ng/ml IL-34 (Peprotech) and 10 ng/ml M-CSF1 (Peprotech) on ultra-low attachment 6-well plates (Corning). Once spheroids with cystic bodies, called Embryoid bodies (EBs) were visible under microscope, EBs were gently triturated to segregate microglia-like cells, and supernatants that contained microglia and microglia

precursor cells were collected and transferred to Primaria 6-well plates (Corning). After 6 consecutive collections of cells, cells were purified by FACS using CD 11b surface markers (Miltenyi Biotec). Purified microglia-like cells were maintained in MGM media with 100 ng/ml IL-34 and 5 ng/ml M-CSF1 for experiments.

Genomic variant analysis for genome-edited iPSCs The sequencing core facility of the Broad Institute of MIT and Harvard handled submitted samples and generated exome-seq data (76-bp paired-end). Our data was processed based on "GATK Best Practices" followed by Broad GATK team guidelines. In summary, the raw fastq files were mapped to human hg19 assembly using BWA mapper (version 7, mem option) (Li and Durbin, 2010); elimination of PCR duplicates were employed by MarkDuplicates function of Picard software package (http://broadinstitute.github.io/picard). Next, local realignment and recalibration were executed using RealignerTargetCreator, IndelRealigner, and BaseRecalibrator modules of GATK tools (McKenna et al., 2010). Using haplotypeCaller of GATK tools, variants in exonic regionas with stand_emit_conf of 10 and stand_call_conf of 30 were called. INDEL variants were chosen using SelectVariants of GATK tools. Genomic variants from genome-edited iPSCs were compared to the variants from their parental lines, resulting in identifying unique variants to genome-edited iPSCS. Variants overlapping with repeatmasker regions and one with low DP were further removed, and then QUAL scores were calculated before functional annotation of exomic variants using ANNOVAR package (Wang et al., 2010). All potential unique variants resulted from the pipeline described above were manually examined by overlaying bam traces of genome-edited iPSCs with their parental lines in IGV

browser (Bobinsone et al., 2011).

Immunoblot analysis iPSC-derived organoids were collected in 1.5 ml tubes. After multiple washing steps with DPBS, the samples were lysed with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholoate, 0.1% SDS, protease and phosphatase inhibitors). After lysates were spun at 13,000 rpm for 15 minutes, supernatants were transferred to new tubes. Protein concentration of samples was measured using Bio-Rad protein assay, and then the equal amount of protein was loaded for electrophoresis.

Measurement of secreted Amyloid β in iPSC-derived neurons by ELISA iPSC-derived neurons were cultured in 96-well plates. After 6 weeks of differentiation, cells were kept for seven days after full media change. Then media were collected and secreted Aβ levels in collected media were measured by human $A\beta_{40}$ or $A\beta_{42}$ ELISA kit.

Amyloid β up-take Oligomerized $A\beta_{42}$ was used for iPSC-derived astrocyte. $A\beta_{42}$ peptide (AnaSpec) was dissolved in 1% NH_4OH with 1 mg/ml concentration, and sonicated. Lysophilized $A\beta_{42}$ was dissolved in water, filtered and incubated at 37 °C for 1 day before any usage. Astrocytes (30,000 cells/well) were seeded in 24-well plates for 2 days and treated with oligomerized $A\beta_{42}$ (250 ng/ml) for another 2 days. Self-degradation levels were measured from oligomerized $A\beta_{42}$ -treated media without astrocytes. The amounts of secreted $A\beta_{42}$ in cultured media were measured by human $A\beta_{42}$ ELISA kit (Invitrogen) following the manufacturer's instructions. Reduced levels of oligomerized $A\beta_{42}$ by astrocytes were calculated by subtracting remaining $A\beta_{42}$ and self-degradation from total $A\beta_{42}$. $A\beta_{42}$ uptake level

was calculated from dividing reduced levels of $A\beta_{42}$ by the number of cells measured by Cell Titer-Glo cell viability assay (Promega). HiLyte Fluor-555 peptide-conjugated $A\beta_{42}$ (AnaSpec) was used for microglia-like cells. The peptide was dissolved in 1% NH_4OH (10 mg/ml) and diluted with PBS (1 mg/ml). Cells were seeded and cultured for 2 days before the experiment, and then treated with the agent (1 μg/ml) for 1 hr for live imaging.

Cholesterol Assay Cholesterol levels in iPSC-derived astrocytes were measured using cholesterol assay kits (Abcam) following the manufacturer's instructions. In order to measure the cholesterol levels, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and treated with filipin III for 1 hour. Fluorescence from cholesterol-bound filipin III was detected by Zeiss LSM 710 confocal microscope. Images were captured using Sen software and analyzed using ImageJ (National Institute of Health) or IMARIS software.

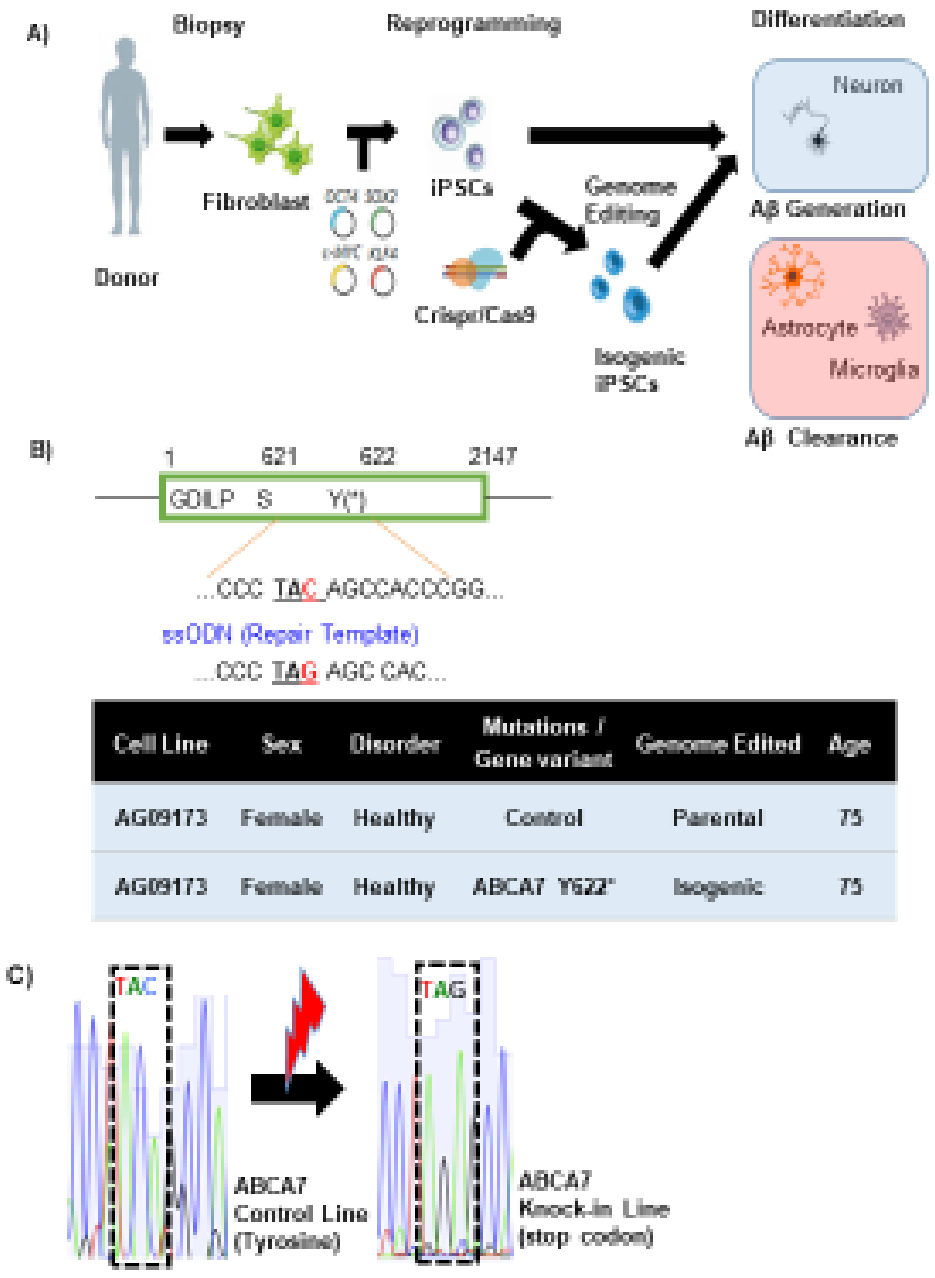
Lysosomal tracking LysoTracker™ Red DND-99 (ThermoFisher) was used to trace lysosomes. After the incubation of 50 nM LysoTracker for 5 minutes, residual lysotracker probes were washed out. Images were captured at intervals of 5 minutes.

Immunostaining analysis 2D culture: differentiated cells were cultured on glass coverslips for this analysis. After DPBS wash-out, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes. After fixation, cells were permeablilized with blocking solution containing 0.1% Triton X-100, 10% donkey serum, 2# BSA and 1 M glycine in PBS for 1 hour at room temperature. Next, cells were incubated with appropriate antibodies overnight at 4 °C and then were treated with

fluorescence-conjugated secondary antibodies including Hoechst 33342 (Invitrogen) for 1 hour at room temperature.

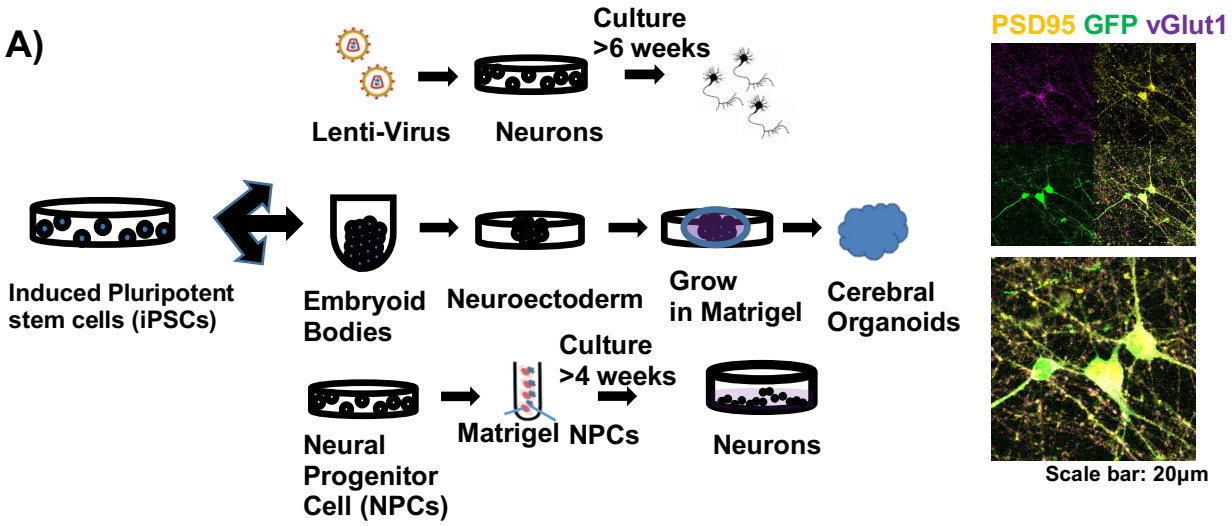
3D culture: organoids were collected and fixed with 4% paraformaldehyde in PBS for 15 minutes. Fixed organoids were further dehydrated with 30% sucrose in PBS at 4 C and then embedded with Optical cutting temperature (OCT) compound (VWR). Embedded samples were solidified by dry ice. The organoids-containing blocks were sectioned with 20 μm thickness using cryosection and transferred to the glass slide. Sectioned organoids were washed with DPBS and permeabilized with blocking solution (0.1% Triton X-100, 10% donkey serum, 2# BSA and 1 M glycine in PBS) for 1 hour at room temperature. Samples were treated with primary antibodies (overnight at 4 °C) and secondary (1 hour at room temperature).

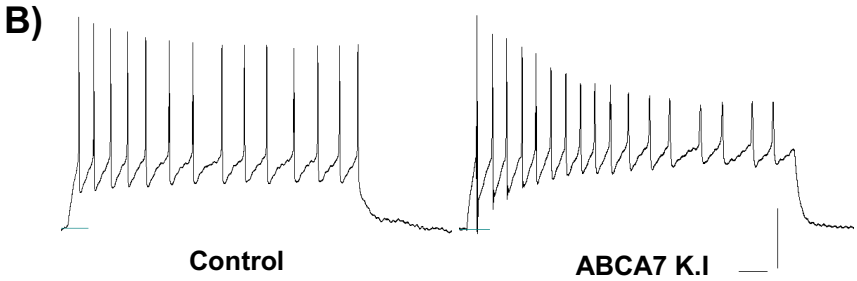
Statistical Analysis Statistical analyses were performed using the software Prism 7 (GraphPad software). ANOVAs followed by Tukey's test, Dunnett's test or unpaired student's t-tests were used. All data are represented as mean ± s.e.m.



Cell Line	Sex	Disorder	Mutations / Gene variant	Genome Edited	Age
AG09173	Female	Healthy	Control	Parental	75
AG09173	Female	Healthy	ABCA7 Y622*	Isogenic	75

Figure 2.1 Generation of isogenic ABCA7 lines from healthy donor. A) Schematic overview of induced pluripotent stem cell (iPSC)-derived Alzheimer's disease (AD) model. B) Target locus for CRISPR/Cas9-driven mutagenesis and information of donor. Tyrosine peptide converted into stop codon guided by single strand Oligooxynucleotides (ssODN) C) Sanger sequencing confirmation on targeted mutagenesis. Single nucleotide replacement from Cytosine to Guanine was made, resulting in conversion from Tyrosine to stop codon.


A)
Culture
>6 weeks
Lenti-Virus
Neurons
PSD95 GFP vGlut1
Induced Pluripotent
stem cells (iPSCs)
Embryoid
Bodies
Neuroectoderm
Grow
in Matrigel
Cerebral
Organoids
Culture
>4 weeks
Neural
Progenitor
Cell (NPCs)
Matrigel
NPCs
Neurons
Scale bar: 20µm


B)
Control
ABCA7 K.I

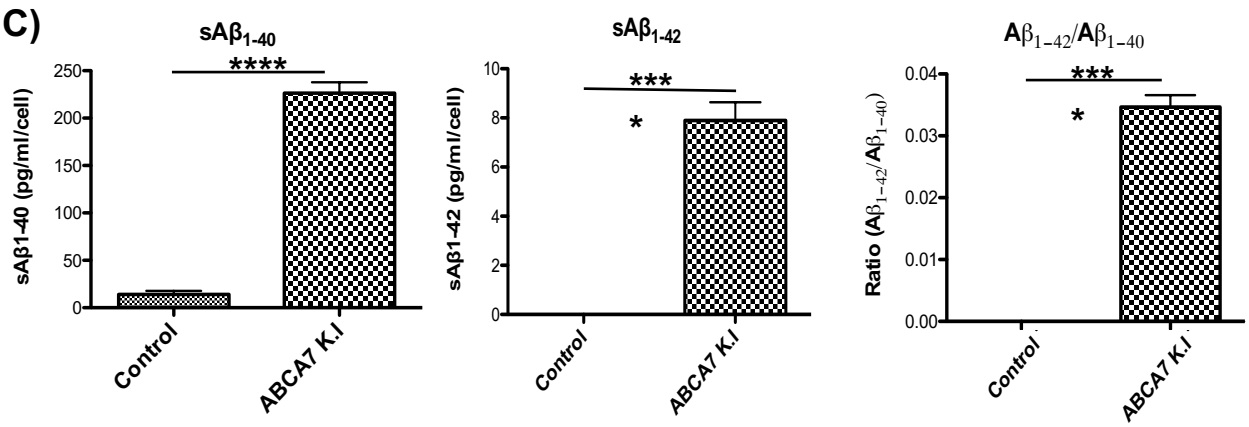

C)
sAβ1-40
sAβ1-42
Aβ1-42/Aβ1-40
sAβ1-40 (pg/ml/cell)
sAβ1-42 (pg/ml/cell)
Ratio (Aβ1-42/Aβ1-40)
****
***
*
Control
ABCA7 K.I

Figure 2.2 Neuronal differentiation from isogenic ABCA7 iPSCs and AD phenotypes recapitulated in iPSC-derived neurons. A) Diverse strategies to induce neural differentiation: 2D neural induction via virus; 3D neural spontaneous induction through self-assembly; and 3D scaffold-based neural differentiation. Neural identity was confirmed by immunohistogram. B) Electrophysiology on induced neurons showing action potential activities. This indicates the maturation of neurons in 6 weeks. C) Increased levels of secreted AD pathogens, amyloid β (Aβ) isoforms measured by ELISA. Both Aβ isoforms were increased in ABCA7 Knock-In (KI) neurons, resulting in increased ratio of $A\beta_{42}$ and $A\beta_{40}$.

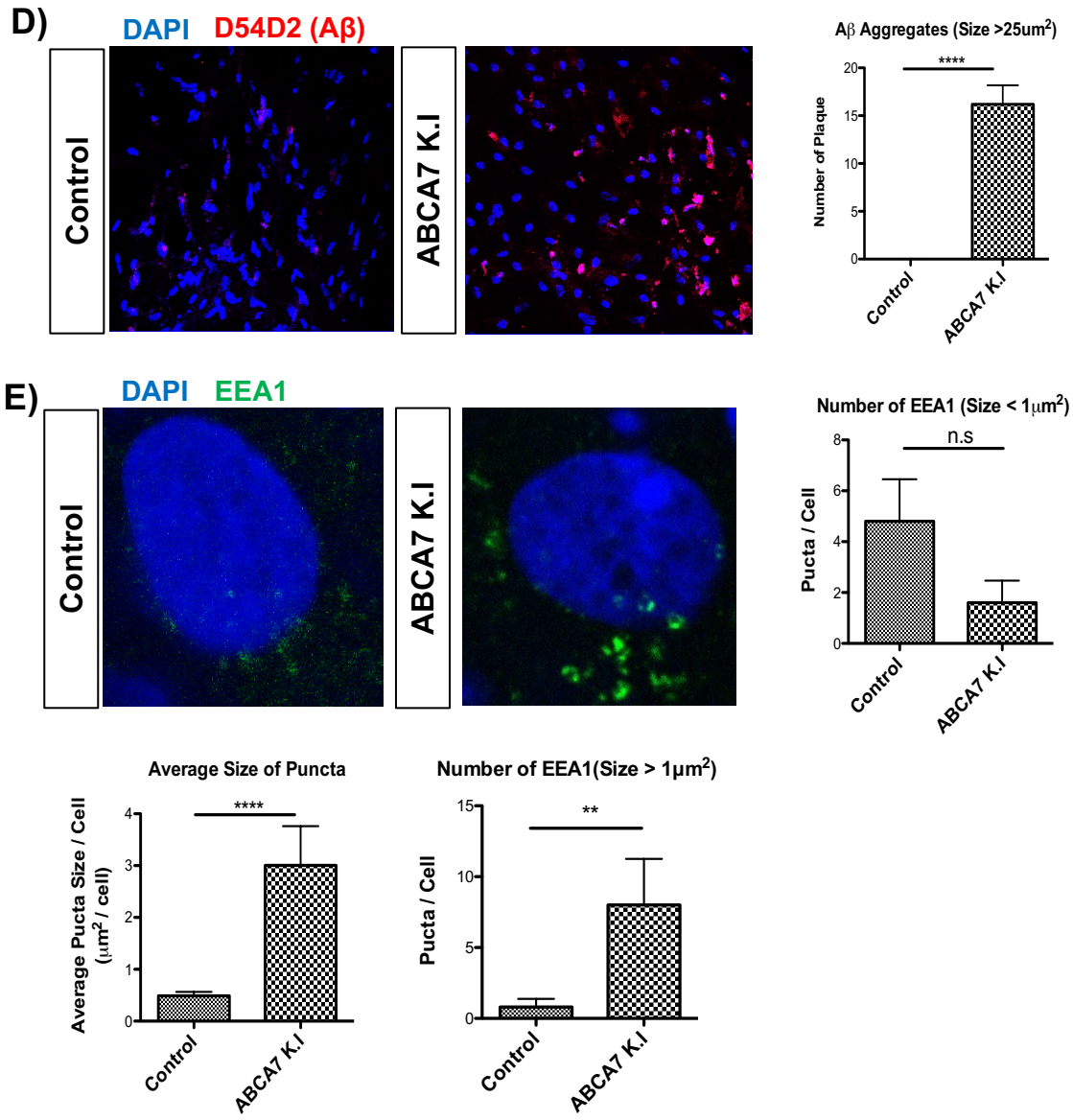


Figure 2.2 (Cont'd) Neuronal differentiation from isogenic ABCA7 iPSCs and AD phenotypes recapitulated in iPSC-derived neurons. D) Presence of Aβ aggregates in 3D scaffold-based neuronal cultures. The immunohistogram confirmed the presence of Aβ aggregates as well as its increased size of them. E) Abnormality in endosomes. Immunohistogram using an antibody against early endosome (EEA1) revealed that ABCA7 K.I neurons contained much more large EEA1+ particles (>1μm^2) with no distinct number of small particles (<1μm^2) compared to the control.

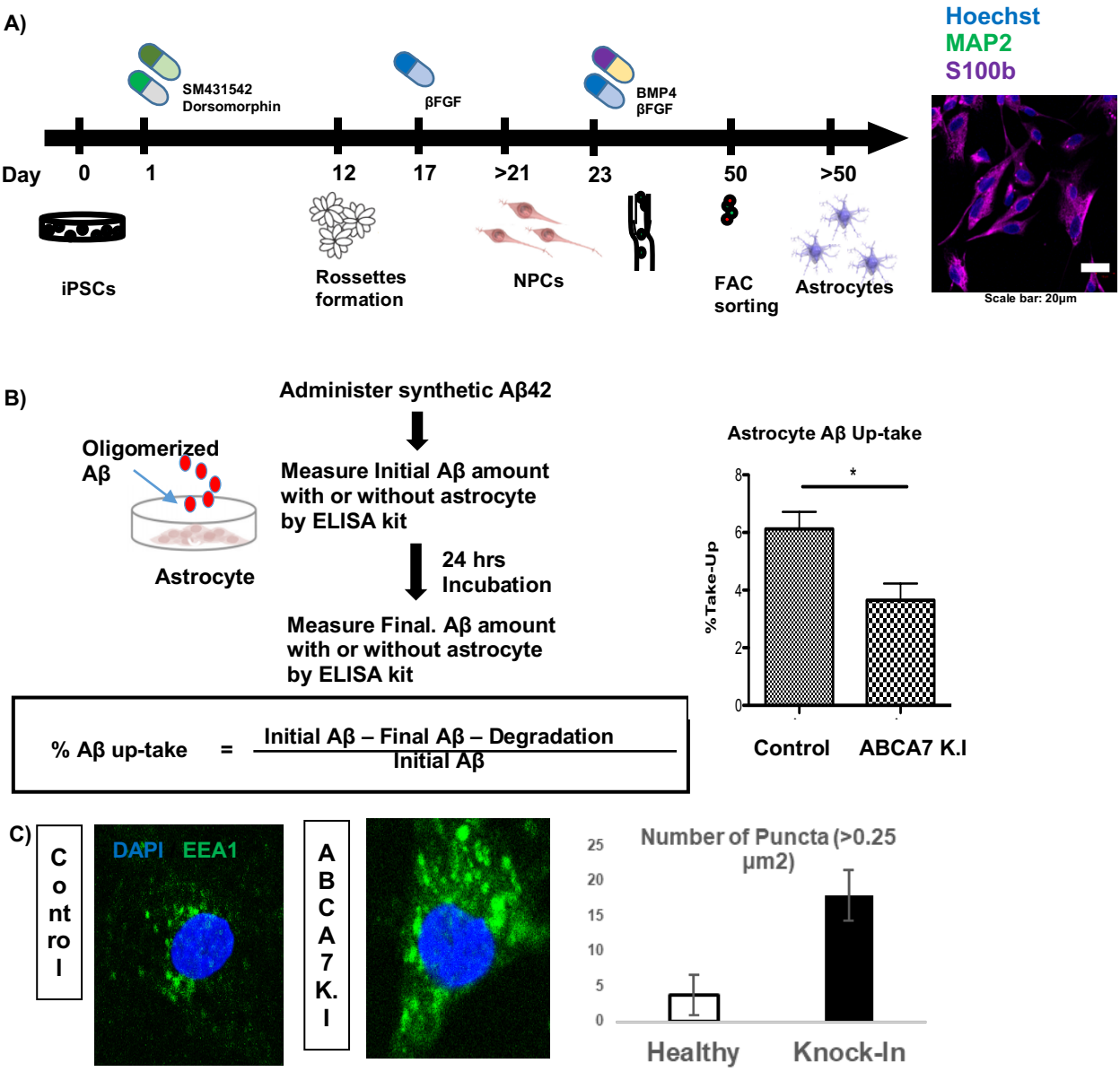


Figure 2.3 Differentiation of iPSCs into astrocytes and relevant AD phenotypes in iPSC-derived astrocytes. A) Overview of two-steps astrocyte inducing procedure using growth factors. iPSCs were driven to induce into neural progenitor cells(NPCs) first as an intermediate cell type, then further differentiated into astrocytes. Their identities were confirmed with astrocyte-specific marker, GFAP. B) Altered Aβ up-take capability of astrocytes. Oligomerized synthetic $Aβ_{42}$s were administered into astrocytes, and residual Aβ levels were measured by ELISA after 24 hours. ABCA7 K.I astrocytes contained less amount of Aβ, indicating impaired Aβ take-up capacity. C) Enlarged early endosomes in astrocytes. Immunohistogram using Early endosome marker 1 (EEA1) showed that the size of early endosomes was increased in ABCA7 KI astrocyte, compared to its isogenic controls.

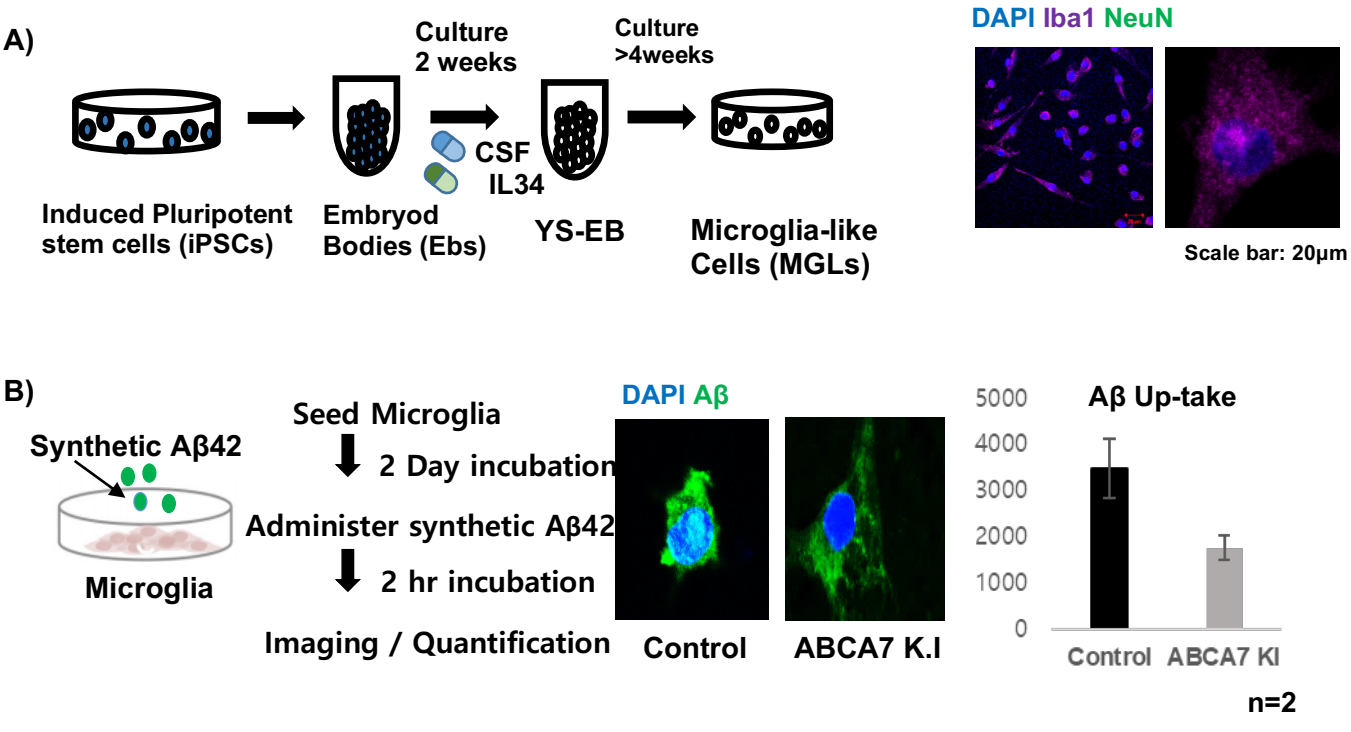


Figure 2.4 Generating microglia-like cells(MGLs) from iPSCs and MGLs-specific AD phenotypes. A) Overview of MGL differentiation protocol. Notably, embryoid body (EBs) were first made, then induced into YS-EB that later produce MGLs via two factors: CSF and IL34. Immunohistogram on these generated MGLs confirmed their microglia-like identities. B) Reduced amount of Aβ uptakes in MGLs. Synthetic $A\beta_{42}$ were administered to test whether ABCA7 K.I could affect the Aβ up-take capability in MGLs. Immunohistogram using an antibodies against Aβ and ELISA results confirmed impaired Aβ uptake capabilities in MGLS. C) Enlarged early endosomes in MGLs. Consistent with results from other brain cell types described above, ABCA7 K.I MGLs also demonstrated the enlarged early endosomes compared to the controls.

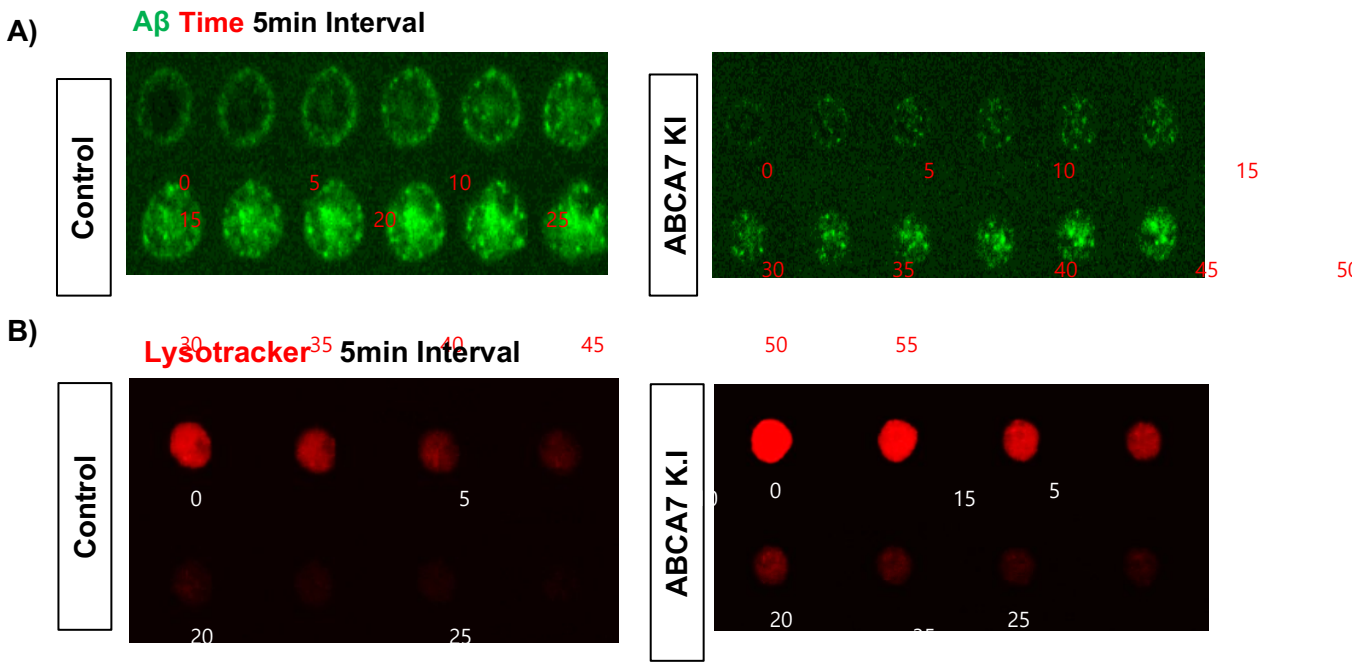


Figure 2.5 Impairment in endocytic trafficking systems. A) Experiments using fluorescence-tagged Aβ demonstrated that ABCA7 K.I MGLs internalized Aβ slowly, resulting less amount of A β inside cells. B) Impaired lysosomal degradation. Lysotrackers were used to estimate the basal activities of lysosome. The data exhibited that lysotrackers dissipitated much slowly in ABCA7 K.I MGLs, suggesting impaired lysosomal activities.

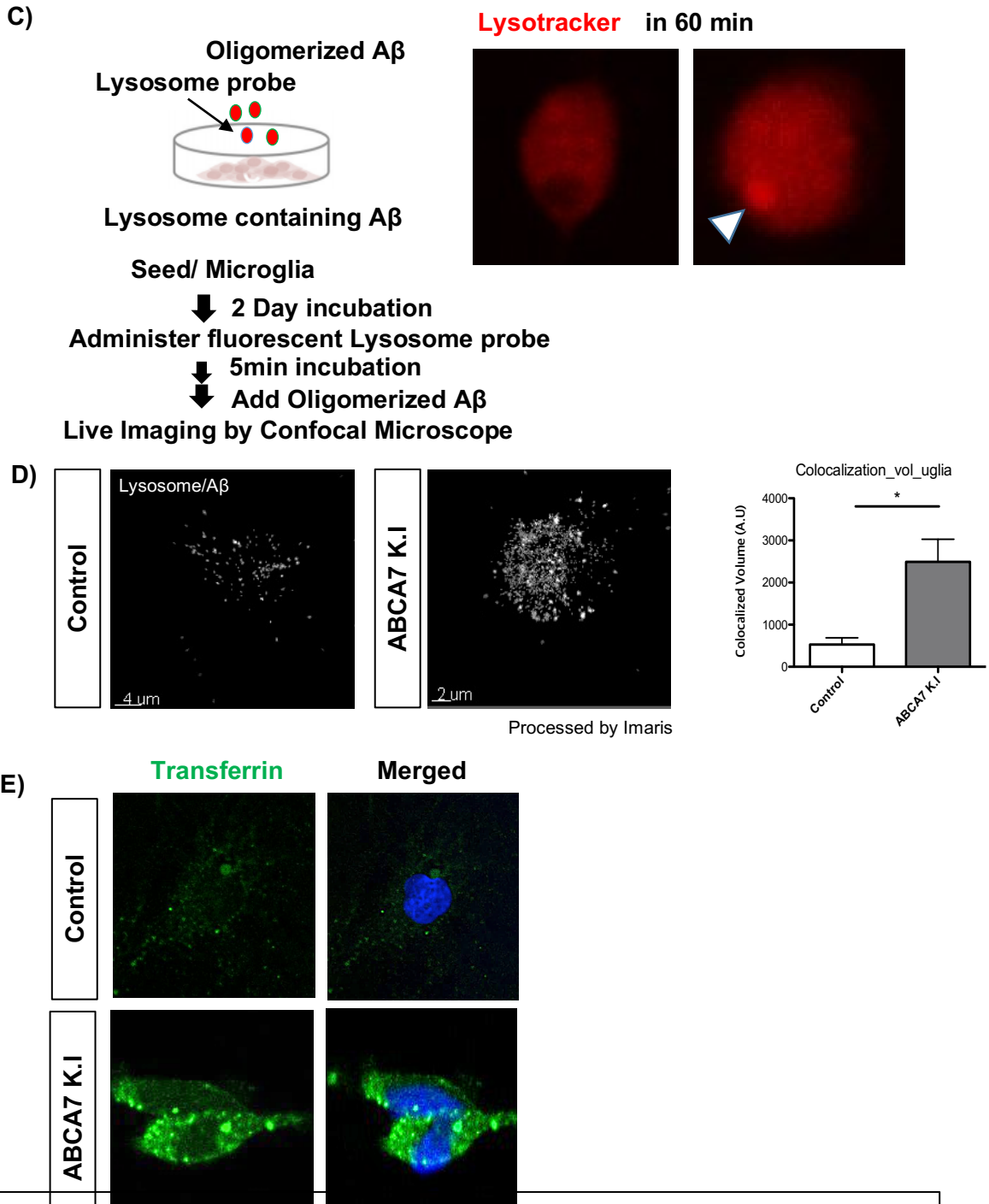


Figure 2.5 ( ) ... ...fficking systems. C) Lysosomal aberrance in MGLs harboring a K.I variant. The lysotracker data revealed the enlarged lysosomes in ABCA7 K.I MGLs after Aβ treatments, compared to the control. D) Increased co-localization of Aβ and lysosome. Experiments using lysotrackers co-treated with Aβ demonstrated that enlarged lysosomes mostly contained Aβ in ABCA7 K.I MGLs. E) Abnormal trafficking in Clatherin-mediated endocytosis. Transferrin data highlighted that the transferrins were aggregated shown as puncta in ABCA7 K.I MGLs.

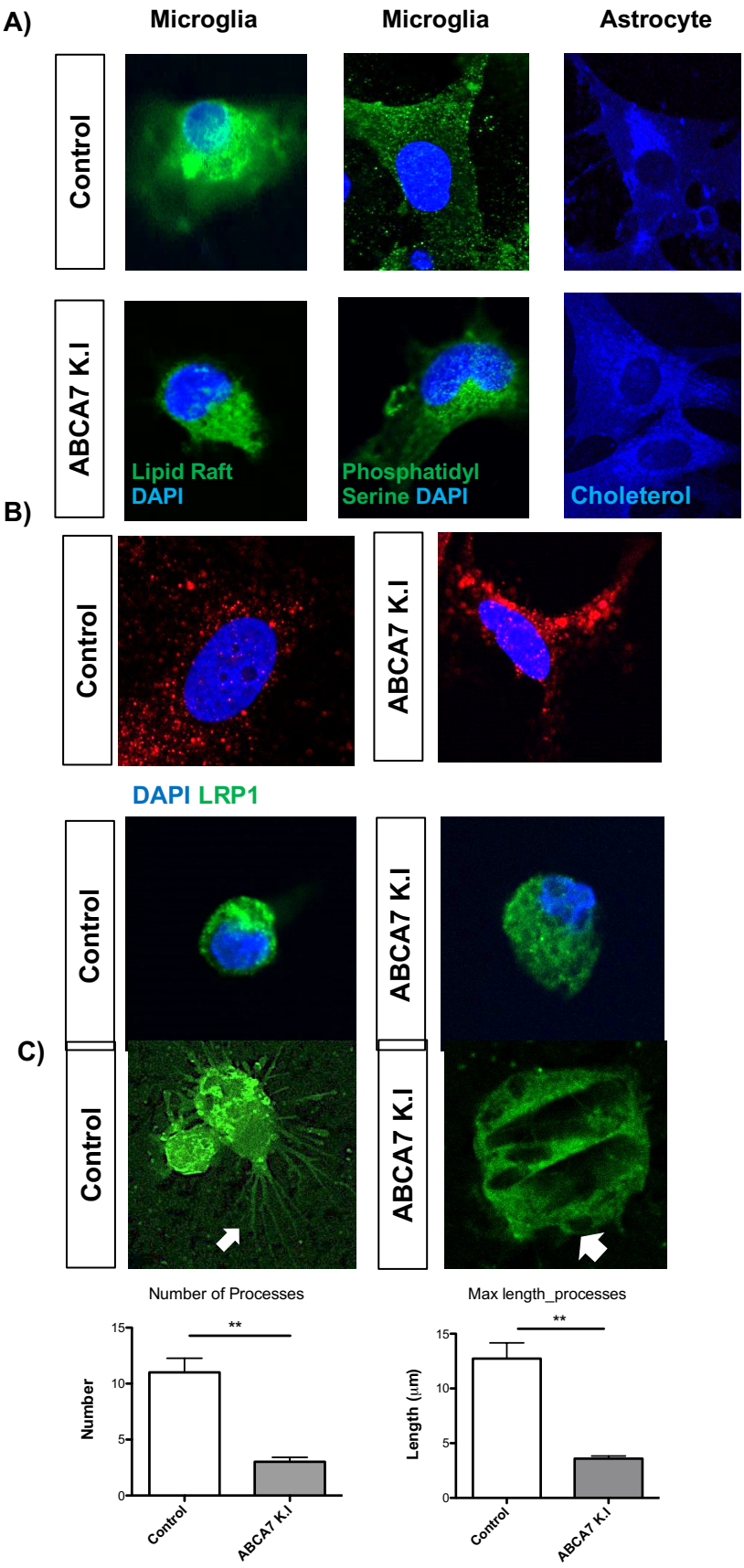

A)
Microglia
Microglia
Astrocyte
Control
ABCA7 K.I
Lipid Raft
DAPI
Phosphatidyl
Serine DAPI
Choleterol
B)
Control
ABCA7 K.I
DAPI LRP1
C)
Control
ABCA7 K.I
Control
ABCA7 K.I
Number of Processes
Max length_processes
Number
Length (μm)
**
**
0
5
10
15
Control
ABCA7 K.I

Figure 2.6 Lipid-related phenotypic alterations and behavioral changes. A) Alterations in lipid compositions. Various kinds of lipids on the membrane were examined. The results highlighted that the concentration and the distributing patterns of tested lipids in ABCA7 K.I MGLs were different from ones in the controls. B) Modifications in membrane-bound proteins. Immunohistogram results exhibited the increased immunoactive signals of ABCA1 in ABCA7 K.I MGLs in contrast to the lower LRP1 levels, compared to the control. The distribution pattern for each protein also changed in ABCA7 K.I MGLs. C) ABCA7 K.I MGLs harbored less number and shorter processes compared to the controls.

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Chapter 3: Engineering three-dimensional *in vitro* Down Syndrome (DS) model using human induced pluripotent stem cell-derived cerebral organoids

Abstract

Down syndrome (DS) also known as trisomy 21 is a chromosomal disorder characterized by brain hypotrophy and intellectual disability. Approximately one in every 700 births worldwide is diagnosed as DS. Despite significant advances in understanding the mechanisms underlying pathological phenotypes via animal models, the limitations of animal models and restricted accessibility to human brain samples make it challenging to study DS and thus to develop therapeutic interventions. With the advent of reprogramming technology, human induced pluripotent stem cells (iPSCs) from an individual with DS create a unique opportunity to investigate DS, but two-dimensional iPSC-based models have limited application in brain disorders by not fully recapitulating features of a brain such as multiple cell types, cellular architectures having dynamic interactions and networks. Furthermore, the broad range of genetic backgrounds between individuals is another challenge to be addressed in human iPSC-based models. Here, we present three-dimensional self-organized human brain structures called cerebral organoids, using DS patient-derived iPSCs (trisomy 21) and its isogenic iPSCs made by spontaneous loss of third chromosome 21 (disomy 21) to investigate DS brain development. We reported that our DS organoid model recapitulate multiple clinically relevant phenotypes. Moreover, we observed that trisomy 21 organoids manifested different CNS developmental patterns compared to disomy 21 orgnoids. We believe that this approach will be useful in investigating the neural development and in modeling developmental brain diseases. It also can provides a more physiologically closer platform for high-throughput screening during drug discovery

for neurodevelopment disorders.

Introduction

Down syndrome (DS), also known as trisomy 21, is a chromosomal disorder harboring an intellectual disability. Approximately one in every 700 births worldwide is diagnosed as DS. The first clinical report on DS was made by Langdon Down in 1866[1,2], and about a century later in 1959, the triplication of human chromosome 21 (HSA21) was reported as the underlying genetic abnormality in DS by Lejeune-Gautier-Turpin[3,4]. The Clear causative roles in DS and the small size make HSA21 the most studied chromosome.

There are three forms of DS: a duplication of HSA21 (94%), translocated HSA21 (4%), and mosaicism (2%)[3,5–7]. Although it is clear that extra copy of HAS 21 causes the pathological conditions and the phenotypes associated with DS, but its molecular mechanisms of DS induction are still unknown. Due to its unknown mechanism, the current treatments are limited to focus on relieving the symptoms, and there is no with no cure.

Traditional approach: animal models but

Among many pathological DS phenotypes, Alzheimer's disease pathology is a well-known feature in DS patients because Amyloid beta precursor protein (APP) gene is located on HSA21. In 1985, AD neuropathological changes were first reported in DS patients[8]. Because Amyloid beta precursor protein (APP) plays significant roles in AD, nearly all DS patients will develop AD pathology starting around age 30. and about 70% will develop dementia at age 55[9]. Furthermore, trisomy 21 (DS) patients comprise the most substantial fraction of people with

early-onset AD (EOAD)[10].

However, despite its comorbidity of AD and DS, these seemingly-different diseases, AD for neurodegenerative disease and DS for neurodevelopmental one, are considered and treated as two different pathologies with the minimal interactions. Along with this rationale, developmental impacts of β amyloids, one of the primary genetic culprits for AD pathologies have not been studied within DS context. Instead, a number of severely manifested Aβ pathologies in DS makes researchers utilize DS animal models as AD models.

Despite these animal model grant tremendous opportunities to investigate DS, however, using animal models has vast limitation caused by interspecies differences. Although many species share some evolutionary constraints in the process to gradually build tissues and organs, called organogenesis, diverging evolutionary needs impose uniqueness to each species. As a result, great understanding how organogenesis is regulated, and how it has changed over the course of time is achieved from plenty of animal models, but knowledge of how human brain develops remains very primitive because of many distinct features of human brain that other species do not have.

Furthermore, serious ethical considerations on access to human brain are challenging to study the human brain itself since the brain is mostly formed *in utero*. Additionally, limited regenerative capacities of a brain provide a restricted platform for investigations because a small piece of a brain (i.e. a live-tissue from microsurgery) cannot be maintained or expanded in the dish.

New approach: iPSC

Technological advances, such as culturing human embryonic stem cells (hES) *in vitro*[11] and reprogramming somatic cells into induced pluripotent stem (iPS) cells[12] combined with directing these stem cells toward a specific cell fate have revolutionized the human brain study, imposing more accessibility to human brain tissues. In particular, three-dimensional structures referred to as cerebral organoids derived from iPSCs have begun to open up a new opportunity to investigate neurodevelopment because previous two-dimensional human cell models cannot recapitulate complicated tissue-level of developmental phenotypes of human brains.

Its very genetic cause of DS, an extra chromosome 21, is another factor to hinder investigation on DS. Despite the clear causation of DS, a considerable amount of genetic variances that each patient has blocked to pin down how this extra chromosome induces DS pathologies. Thus, minimizing genetic backgrounds becomes a great deal in designing experiments. However, scarce isogenic DS sources (e.g. monozygotic DS twin, mosaic DS) and the technical difficulties to modify the entire chromosome, impede researchers to draw clinically meaningful information from studies.

iPSCs can be not a replacing model for animal models, but a good complement model

Despite the availability of human stem cells, poor accessibility to all stages of development as well as lack of functional tissue preparations still makes the study of human brain challenging. Although previous post-mortem studies provide

information on structural brain development, they are, however, limited by sample size and cross-sectional design[13]. There are advanced imaging tools, such as multi-shell diffusion-weighted MRI and diffusion-weighted imaging[14,15] available for brain structural studies, but their imaging resolutions cannot provide cellular level structural information. In particular, post-mortem histological and gene-expression studies show that 35% of adult brain volume is formed by 2-3 weeks after birth[16], reaching about 80% of the adult size in the second year of the life[16]. Structural growth proceeds followed by network maturation in the cortical grey-matter region[17], suggesting the early establishment of fundamental brain structure and functions. Brain development at the early stage is also critical for risk of brain diseases, such as autism spectrum disorder and schizophrenia[18]. Despite its significance, however, little is known about the structural and functional development of the brain during this period.

Therefore, it is necessary to develop functional models of the developing human brain to understand the underlying principles for disease mechanisms. Understanding its unique disease biology can provide valuable mechanistic insights, which may lead to therapeutic advances.

Recently, Lancaster et al. show that human iPSC-derived three-dimensional organoid culture system, called human cerebral organoid nicely recapitulates several discrete human brain developmental features, such as cellular diversity including radial outer glial cells and regionalized developments[19]. Further characterization of cerebral organoids by performing single-cell RNA-seq with the comparison to previously published human data, confirms that its genomic profile

was similar to one of the human fetal cortical brain[20], suggesting unprecedented accessibility to limited brain sample and opportunity to study aspects of human brain disease.

In this chapter, we utilized human iPSCs derived from DS patient and its isogenic line of iPSCs created by a spontaneous loss of extra chromosome that DS patient-derived iPSCs have. Using these isogenic iPSC lines, we established human iPSC-derived DS cerebral organoids and explored how developmental progress can be influenced by extra chromosome. We also examined if Aβ pathologies induce any developmental effects and vice versa.

Results

Although many studies describe that iPSCs trisomy for chromosome 21 have stable karyotypes[21–23], Daley et al. reported the emergence of isogenic di- and trisomic iPSCs from a mixed population due to the spontaneous loss of extra chromosome[24]. Due to minimal variations in genetic backgrounds, we utilized these isogenic lines. Firstly, we tested the karyotypic stability of these lines. Our karyotype analysis results describe that no other chromosomal abnormalities were found in any subclonal iPSCs of both lines. (Figure 3.1B). We also performed pluripotency test on both lines by immunofluorescence using antibodies against Tra1-60 and Oct4, pluripotent markers and confirmed that both di- and trisomic iPSCs do not have aberrant pluripotent phenotypes. Next, using built protocol by Lancaster et al., we tested whether DS isogenic iPSCs have a self-organizing capacity to form brain-like tissues, called cerebral organoids that develop various discrete brain regions (Figure

3.1A). We confirmed that both lines could develop multiple intermediate developmental structures such as neuroectoderm and neuroepithelia necessary for the brain tissue formation (Figure 3.1C). Interestingly, though cerebral organoids from both lines were established within 20-30 days, we noticed that trisomy 21-cerebral organoids have several distinct abnormalities. First, trisomy 21 iPSCs were relatively hard to derive into cerebral organoids whereas the isogenic disomic 21 iPSCs had almost one hundred percent of success in forming desired structures (Figure 3.1D, Left). This abnormality in cerebral organoids formation was also detected in other DS patient-derived iPSCs that were created independently. In many cases, trisomy 21 failed to develop into neuropethilia, resulting in degradation (Figure 3.1D, Right). Additionally, it was observed that the morphology of neuroepithelia in cerebral organoids derived from trisomy 21 iPSCs was different from ones from one in disomy 21.

DS cerebral organoids manifest phenotypes found in DS patients

One of the biggest downfalls in animal models is a limitation to recapitulate pathological phenotypes observed in human brain disorders. Therefore, we tested whether DS cerebral organoids could be used to model Down syndrome. Microcephaly is one of the developmental deficits DS patients have. It is a rare neurological condition and usually caused by abnormal brain development in early stages such as in utero and after birth. We decided to test whether such pathological condition could be recapitulated in the cerebral organoids model. Based on our observation that the diameter of embryoid bodies and observed smaller embryoid

bodies in DS condition, which often led to failure in further development, and that the short stature is a cardinal sign of DS[25], we tested whether trisomy 21 could perturb further embryoid body growth. For this test, we performed 30 days long cerebral organoids culture and measured the diameter of cerebral organoids. Our data revealed that smaller neuroepithelial tissues were generated with DS iPSCs, indicating the reminiscence of the reduced brain size found in DS patients (Figure 3.2A). The histological analysis suggested that human cerebral organoids displayed a progressive reduction of pluripotency as neural induction initiated during organoid differentiation[19]. To examine whether the decreased efficiency of initial neural induction resulted in the smaller size of DS cerebral organoids, we performed quantitative PCR (qPCR), also known as real-time PCR for pluripotent markers OCT4 and Nkx2.1. As expected, we detected diminished levels of both pluripotent markers OCT4 and Nkx2.1 in DS cerebral organoids, indicating less capability for neural induction (Figure 3.2B). This result might suggest why DS cerebral organoids did not reach the similar size of disomy 21 tissues due to the lack of cell sources for neural induction. During the brain development *in vivo*, it exhibited several striking features such as heterogeneous regionalization and the regional interdependence. Lancaster et al. also showed that the similar degree of brain regional heterogeneity could be recapitulated in cerebral organoids[19]. To test whether our DS cerebral organoids model could recapitulate any deficits shown during early brain regionalization in the whole brain, we performed qPCR for forebrain (SIX3 and FOXG1), midbrain (LMX1B) and spinal cord (HOXB4) markers. Interestingly, our data exhibited that the patterns of brain regional development in DS cerebral

organoids were different from ones in isogenic organoids, and also revealed that those corresponding populations were present within the tissue (Figure 3.2C). Isogenic disomy 21 cerebral orgnoids exhibited higher levels of expression in forebrain markers relative to ones for midbrain and spinal cord, reflecting the expansion of forebrain region during human brain development[26]. In contrast to disomy 21, DS cerebral organoids showed the lower expression in forebrain markers but with an increased level of markers for midbrain and spinal cord, reminiscent of abnormal brain development of DS forebrain[27,28].

AD phenotypes captured in DS cerebral organoids model

Due to the role of amyloid beta precursor protein (APP) on AD pathologies[29], Alzheimer's disease pathology is one of well-known features in DS patients. We tested whether DS cerebral organoids could perpetuate this phenotype. We performed immunohistochemistry with an anti-Aβ antibody, D54D2 recognizing various isoforms of amyloid ($A\beta_{37}$, $A\beta_{38}$ $A\beta_{40}$, and $A\beta_{42}$) and aggregated Aβ aggregates. Once we detected aggregates immunopositive for Aβ antibody in DS cerebral organoids, we quantified D54D2-immunopositive signals to measure the size and number of Aβ aggregates. Our analysis demonstrated that the increase in size and number of Aβ aggregates was identified in DS cerebral organoids compared to the isogenic disomy 21 organoid tissues (Figure 3.3A). To test further Aβ pathology, we subjected culture media from DS cerebral organoids and isogenic disomy 21 cerebral organoids to ELISA. Consistent with the previous study and the immunostaining result, higher levels of both $A\beta_{40}$ and $A\beta_{42}$ were detected in culture

media from DS organoids in contrast to the lower level of the ratio of $A\beta_{42}$ and $A\beta_{40}$ (Figure 3.3B). Thus, our various measures support that Aβ phenotypes can be mimicked in DS cerebral organoids.

Another hallmark of AD is tauopathy, aberrant hyper-phosphorylated tau protein aggregates (pTau). Neurodegeneration is led by β-sheets of pTau via defects in the neuronal microtubule assemblies[30]. To determine whether tau pathology was present in DS organoids, we performed immunohistochemistry using an antibody against pTau (Ser396). The DS organoids exhibited greater pTau immunoactivity than the disomy 21 organoids (Figure 3.3C). Furthermore, we performed the Western blot on whole organoids lysates and detected increased levels of pTau in DS organoids compared to isogenic disomy 21 organoids (Figure 3.3D).

Abnormalities in endosome are another well-known phenotypes found in AD patients[31]. In DS patients, early endosomes are significantly enlarged in some pyramidal neurons as early as 28 weeks of gestation, decades before classical AD neuropathology develops[32]. To examine endosome phenotypes in cerebral organoids, we performed immunocytochemistry with an antibody against the early endosome marker 1 (EEA1) and detected that DS organoids exhibited higher number of large endosomes (>1μm^2) compared to isogenic disomy 21 organoids, whereas the number and size of small endosome (>1μm^2) were indistinguishable between DS and disomy 21 organoids (Figure 3.3E). We performed Western blot on lysate from both organoids to further examine aberrant endosome, and observed increased levels of endosomes in DS organoids compared to the disomy 21 organoids (Figure 3.3F). Incomplete degradation of macromolecules in lysosome results in the

accumulation of intermediate in endocytic trafficking system[33]. Thus, we performed the Western blot to investigate whether the presence of the abnormality in lysosome existed in DS organoids. Our analysis demonstrated the increased level of lysosome in DS compared to one in disomy 21 organoids (Figure 3.3G). Together, we highlight that DS cerebral organoids can robustly recapitulate the AD pathological phenotypes such as Aβ, pTau, and endosomes.

Attenuating pathologies that DS organoids recapitulate by facilitating neural induction

Proliferation deficits are identified both in DS animal models and in DS patients[34,35]. In previous data, we observed that DS embryoid bodies fail to develop further when transferred to neural induction, resulting in the smaller tissues. Thus, we proposed that the slower proliferation induced disruption in overall growth and CNS development pattern. Therefore, we tested whether deficits in CNS development in DS cerebral organoids could be rescued by extending neuronal induction periods that in turn result in producing more cells ready to differentiate (Figure 3.4A). Our qPCR data with markers used previously revealed the complete disappearance of pluripotency in DS cerebral organoids (Figure 3.4B). We further tested the CNS development patterns by qPCR and observed the complete loss in corresponding CNS populations (Figure 3.4C). Together, the data suggest that extended neural induction period could not alleviate the limited capability of neural differentiation, resulting in deficits in the developmental pattern in DS cerebral organoids.

Pharmacological perturbations alleviates DS developmental deficits

Our data demonstrated that both relevant DS- and AD-like phenotypes were mimicked in DS cerebral organoids. In order to determine whether these phenotypes can be attenuated by any available pharmacological interventions, we subjected cerebral organoids to these compounds: Epigallocatechin-3-gallate (EGCG) and a BACE-1 β-secretase inhibitor. EGCG, a green tea flavanol is a kinase inhibitor[36]. Recent clinical data have pointed that EGCG can be a potential treatment for DS patients[37,38]. BACE-1 β-secretase inhibitor is to inhibit activities of β-secretase that generates Aβ by APP cleavage, thereby resulting in alleviating AD pathologies. The drug treatment was begun on day 7 when corresponds to the neural induction period (Figure 3.5A). Strikingly, our data exhibited that DS cerebral organoids treated by BACE-1 β-secretase inhibitor had the increased size of diameter compared to DS organoids treated with DMSO vehicles while DS organoids treated with EGCG has increased, but insignificant improvements in diameter (Figure 3.5B). The effects of both compounds on pluripotency were tested, and the data highlighted that EGCG facilitated pluripotency only in disomy 21 organoids, whereas little effect on pluripotency was observed in DS organoids (Figure 3.5C Left). On the other hand, the data from BACE1 β-secretase inhibitor showed that the enhanced pluripotency was detected only in DS cerebral organoids while no additional facilitation was made in disomy 21 cerebral organoids (Figure 3.5C Right). We further investigated how these drugs affect CNS developing profiles observed above. Our qPCR results revealed that EGCG could not modulate abnormal patterns in CNS development whereas it could enforce the existing CNS development pattern of

disomy 21 cerebral organoids (Figure 3.5D). On the other hand, our results exhibited the efficacy of BACE1 β-secretase inhibitor on selected genes, SIX3, LMX1, and HOXB1, associated with development in forebrain, hindbrain and spinal cord, respectively whereas the negative effect on another forebrain development-associated gene, FOXG1 (Figure 3.5E). Together, our data highlighted that DS cerebral organoids have the different drug-responding profile in developing genes, compared to the disomy 21 cerebral organoids. Besides, our study revealed that deficit in DS cerebral organoids could be modified by perturbations, suggesting the potential of our model as a therapeutic drug-screening platform.

Discussion

Early childhood between birth and two years of age is critical for cognitive and behavioral developments[39]. Increasing pieces of evidence show that this period is strongly associated with neuropsychiatric disorders, such as autism spectrum disorder and schizophrenia[18]. Several studies show that fundamental structural and functional brain development are established in rapid pace by the second year of life[14,16,40,41]. Recent post-mortem histological and genetic studies strongly support these findings by showing the basic framework of brain structure and functions is in place during first two years or earlier, and then reorganization of pre-formed circuits and networks occurs afterward[42,43]. Despite its significance, structural and functional development of the human brain during this time window remain unknown in part because of limited accessibility to the tissues and poor resolution of imaging tools. Therefore, the most knowledge on the brain development heavily

relies on the studies of embryogenesis *in vivo* with various animal models. In order to overcome aforementioned limitations of animal models, we engineered a three-dimensional self-assembled brain structure called a whole brain cerebral organoid using DS patient-derived iPSCs and their isogenic disomy 21 lines, respectively.

EGCG is one of the promising therapeutic tools for DS patients[44]. Several mice studies showed that adaptive functionality and cognitive deficits associated with DS were improved by EGCG treatments[37,45,46]. Furthermore, a range of clinical data supported the efficacy of EGCGs on adaptive functionality and cognition[37,38,47].

While animal models provide invaluable insight and knowledge on the brain development, yet, it is well known that animal models could not recapitulate many traits of human neurological diseases in animal models, thereby providing limited ability to study human disease mechanisms. These limitations, in turn, result in the bottleneck for drug development processes. In this study, using DS patient-derived cerebral organoids, we successfully recapitulated pathological phenotypes manifested in DS patients: developmental and β amyloid pathologies. Interestingly, our data highlight that DS cerebral organoids can displayt those phenotypes as early as 30 days while it takes 60-90 days to recapitulate AD pathologies in cerebral organoids modeling for familial AD[48]. This early manifestation can be beneficial in two ways: to reduce financial costs in stem cell research, and to facilitate research pace with faster turnover, both which are impeding factors in stem cell field.

Besides, we demonstrated that these phenotypes were modifiable by pharmacological interventions. These results suggest the enormous potential of this model as a drug-screening platform for human neurological diseases. Strikingly, we

noticed that BACE-1 β-secretase inhibitor could attenuate several deficits mimicked in DS cerebral organoids. This result proposes the possibility of repurposing β-secretase inhibitor whose recent clinical trials on AD treatment went failed. Altogether, this study demonstrates the great promise of the iPSC-derived DS model using cerebral organoids as a platform both for a mechanistic study and for a drug-screening purpose.

Experiments Procedure

Human Induced Pluripotent Cells (hiPSCs) Cultures Human DS isogenic iPSC lines were kindly shared by Daley laboratory. iPSCs were cultured and maintained either on Matrigel (BD Biosciences) with mTeSR1 media (Stem Cell Technologies) or on irradiated mouse embryonic fibroblasts (MEFs, MIT-GlobalStem) in human ES (hES) media containing DMEM/F12, HEPES media (Gibco) supplemented with 20% knockout serum replacement (KSR, Gibco), 1X non-essential amino acids (NEAA), 1X GlutaMax (Life Technologies), 12 nM β-bifroblast growth factor (β-FGF2, PeproTech) and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich). iPSCs were maintained at 37 ˚C and 5% CO_2 in a humidified incubator.

Karyotyping In order to evaluate the size, shape, and number of chromosomes in disomy and trisomy iPSCs, we performed karyotyping. iPSCs were cultured on Matrigel (Corning) in mTESR1 media (Stem Cell Technologies) and maintained until its confluence reached to 80%, and then sent to Cell Line Genetics for the analysis.

Generation of Cerebral Organoids The generation of cerebral organoids

from iPSCs was performed as previously described with slight modifications (Lancaster et al., 2013). In brief, for embryoid bodies (EBs) formation, iPSCs were transferred into 96-well plates with cone-shaped wells (Nucn1 96-well Conical Bottom plates, VWR International). Media for EBs culture contained that DMEM/F12 supplement with KSR (20% v/v), Sodium Pyruvate (1X), NEAA (1x), 2-mercaptoethanol (0.1mM) and Rock inhibitor (20 μM). βFGF was kept in EB media for first four days, and absent in day 5 and 6. On day 7, full media replacement was made with neural induction media containing DMEM/F12 supplemented with GlutaMax (Invitrogen), N2 supplement (Invitrogen), nonessential amino acids (NEAA) and 1 μg/ml heparin (Sigma) for next four days. Media was replaced every day. When translucent edges were observed in EBs, EBs were encapsulated within Matrigel (final 1% v/v, Corning Incorporated—Life Sciences), and then maintained with new media of the 50:50 mixture of DMEM/F12 and Neurobasal containing N2 supplement, B27 without Vitamin A (Invitrogen), 2-mercaptoethanol, insulin (Sigma), GlutaMax and NEAA for four days. Since then, B27 without Vitamin A was replaced with B27 with Vitamin A (Invitrogen) and kept throughout the culture period.

Cryosectioning and Immunostaining Analysis four – five 30 day-old cerebral organoids were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) and cryoprotected in a 30% sucrose solution. Fixed tissues were embedded in optimal cutting temperature compound (OCT, VWR international) and solidified by dry ices. Using a cryostat, frozen tissue was sectioned with 30 μm thickness, and mounted on ultra-frosted glass microscope slides. Sectioned tissues

were stored at -20 °C before immunostaining. For immunolabeling, tissues were first treated with phosphate-buffered saline (PBS) containing 0.3% Triton-X 100 (Sigma-Adrich) for permeabilization. Tissues were further treated with the blocking solution containing 10% v/v goat serum supplemented with 0.1% Triton-X 100 for 1 hour. Primary and secondary antibodies were prepared in PBST containing 5% goat serum. Tissues were incubated in primary overnight at 4°C followed by 1 hour long secondary incubation with appropriate washing steps. Then fluoromount-G (Electron Microscopy sciences) was mounted on sections. The primary antibodies were used with following diluting ratio: MAP2 (1:400, Biolegend), β-amyloid (D54D2) (1:400, Cell Signaling Technology), phosphorylated Tau, Ser396 (1:400, Cell Signaling Technology) and Early endosome antigen 1 (EEA1) (1:500, BD Biosciences)

Immunoblot Analysis Eight to ten cerebral organoids were collected in 1.5 ml tubes. Multiple washing steps were performed with DPBS to remove residual culture media. Next for protein extraction, RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholoate, 0.1% SDS, protease and phosphatase inhibitors) was used to lysate samples. Then, lysates were spun at 13,000 rpm for 15 minutes; supernatants were transferred to new tubes. Protein concentration of samples was measured using Bio-Rad protein assay, and then the equal amount of protein was loaded for electrophoresis.

Amyloid β ELISA secreted soluble $A\beta_{40}$ and $A\beta_{42}$ concentrations for di- and trisomy cerebral organoids were measured from each supernatants of corresponding collected organoid media using ELISA kit for human $A\beta_{40}$ and $A\beta_{42}$

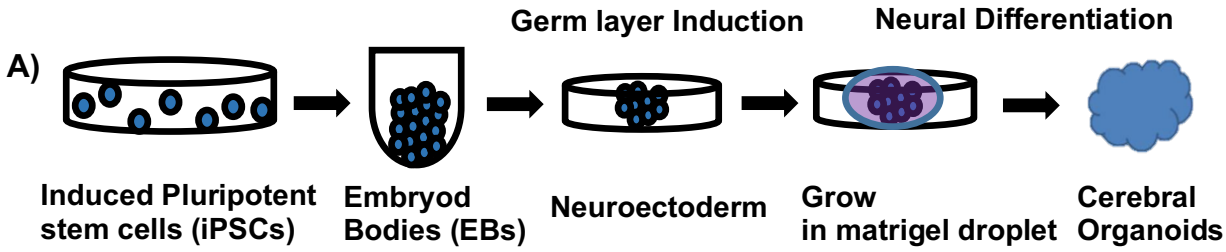
(Life Technologies Corporation) following the manufacturer's protocol. In short, media samples were collected and incubated overnight at -4 °C in in pre-coated 96 well plates with monoclonal antibody specific to human Aβ 1–40 or 1–42, followed by secondary antibodies and addition of HRP substrate. After stopping the reaction with stop buffer, absorption was measured at 450 nm by a plate reader (EnSpire Perkin Elmer).

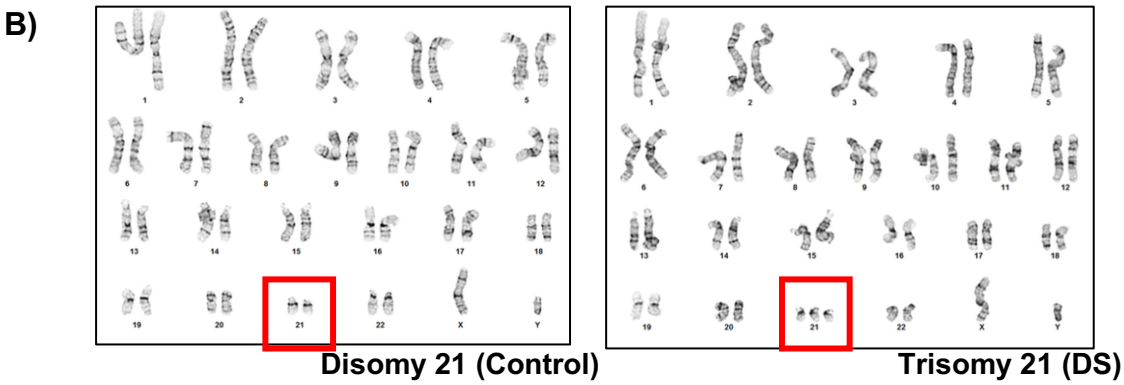
Image processing Sectioned organoids were imaged by a laser scan confocal microscope (LSM880, Carl Zeiss). Images were further processed using ImageJ software (NIH) for quantification. Measurements consisted of counts for the number of particles and size for β-amyloid (Aβ), EEA1, and transferrin immunoreactivity, as well as signal intensity for pTau. Particle count and size were measured in following manners: first, convert the desired image channel into grayscale with automatic thresholding, then invert image, and execute the Analyze Particle measurement tool. Particle counts were binned by size range. For pTau immunoreactivity, CTCF (the mean intensity – background) was measured.

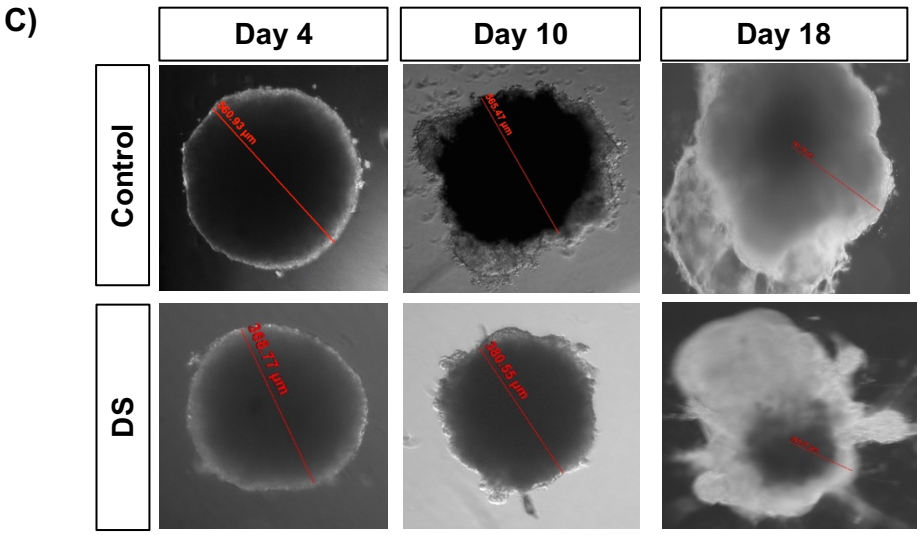
Drug treatments Cerebral organoids were subjected to drug treatment for >25 days with beta secretase (BACE-1) inhibitor (β-Secretase Inhibitor IV, EMD Millipore) and epigallocatechin-3-gallate (EGCG, Sigma-Aldrich) or equivalent DMSO vehicle. Each compound was administered in 5 μM or 10 μM, respectively after dilution from a 5mM DMSO stock. The same concentration of DMSO vehicle was used for vehicle-treated cultures. Drug treatments had begun at day 7 of neural induction until the endpoint at 30 days of culture.

Statistical Analysis All statistical analyses were conducted using the

software Prism 7 (GraphPad software). Unpaired student's t-tests were used. All data are represented as mean ± s.e.m.


A)
Germ layer Induction
Neural Differentiation
Induced Pluripotent stem cells (iPSCs)
Embryod Bodies (EBs)
Neuroectoderm
Grow in matrigel droplet
Cerebral Organoids


B)
Disomy 21 (Control)
Trisomy 21 (DS)


C)
Day 4
Day 10
Day 18
Control
DS

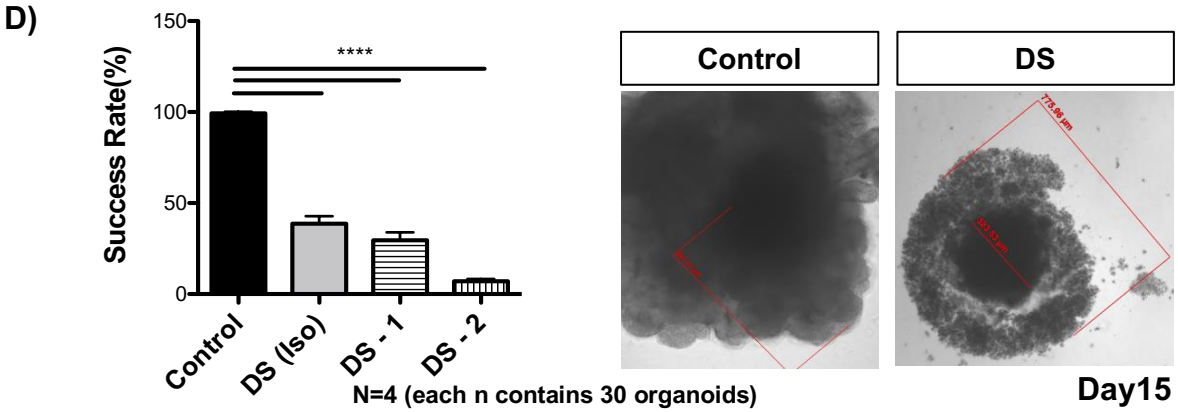

D)
Success Rate(%)
150
100
50
0
****
Control
DS (Iso)
DS - 1
DS - 2
N=4 (each n contains 30 organoids)
Control
DS
Day15

Figure 3.1 DS cerebral organoids have difficulties in deriving into cerebral oragnoids. A) Schematic overview of making cerebral organoids. B) Karyotyping analysis exhibited the number of chromosome 21: disomy 21 (Left), trisomy 21 (Down syndrome) (Right). C) Bright filed images of disomy 21 and trisomy 21 cerebral organoids. D) DS cerebral organoids lad lower success rate into cerebral oragnoids formation. While developing into ogrganoids, DS iPSCs often failed to further develop into neuroectoderm.

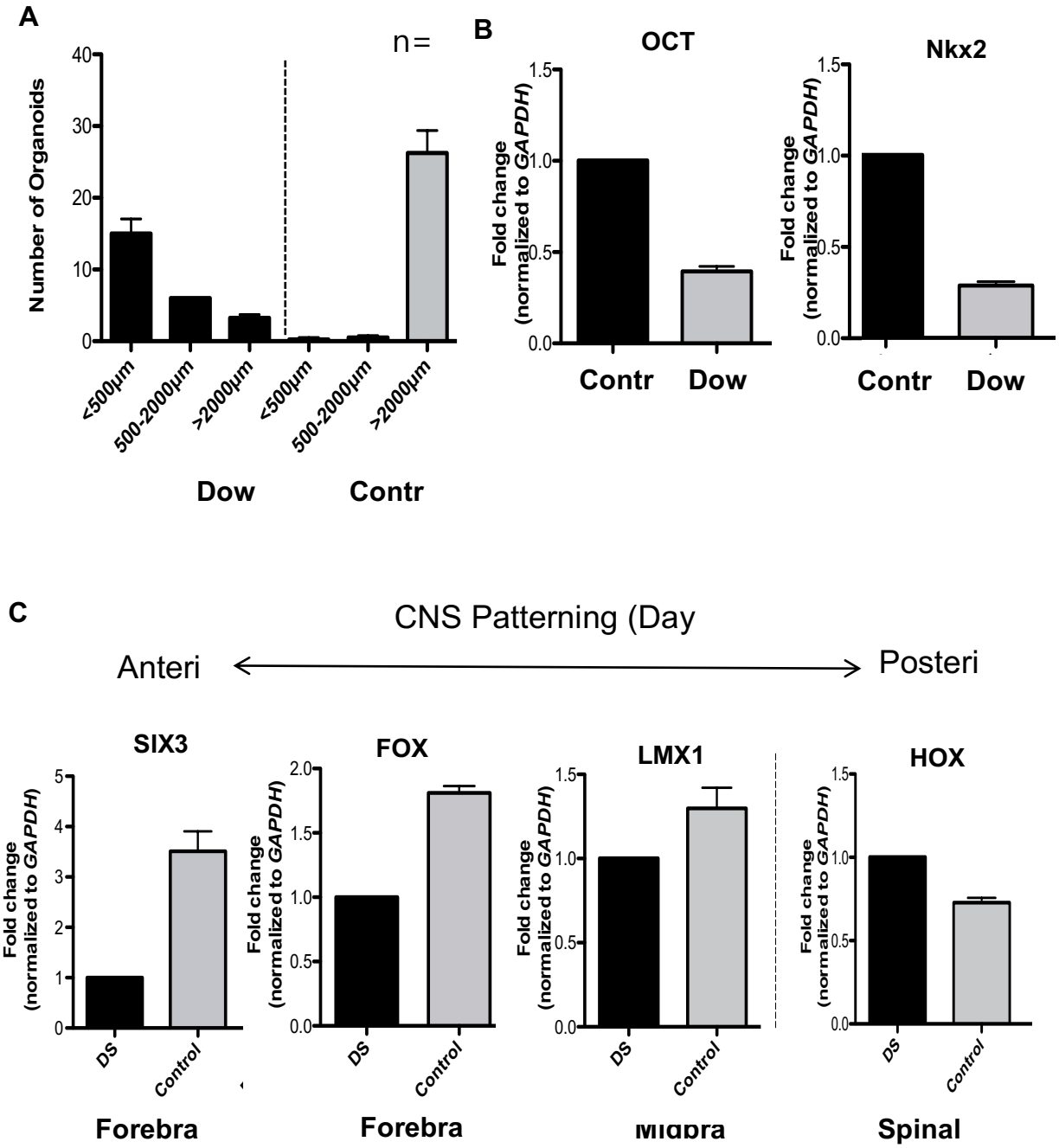
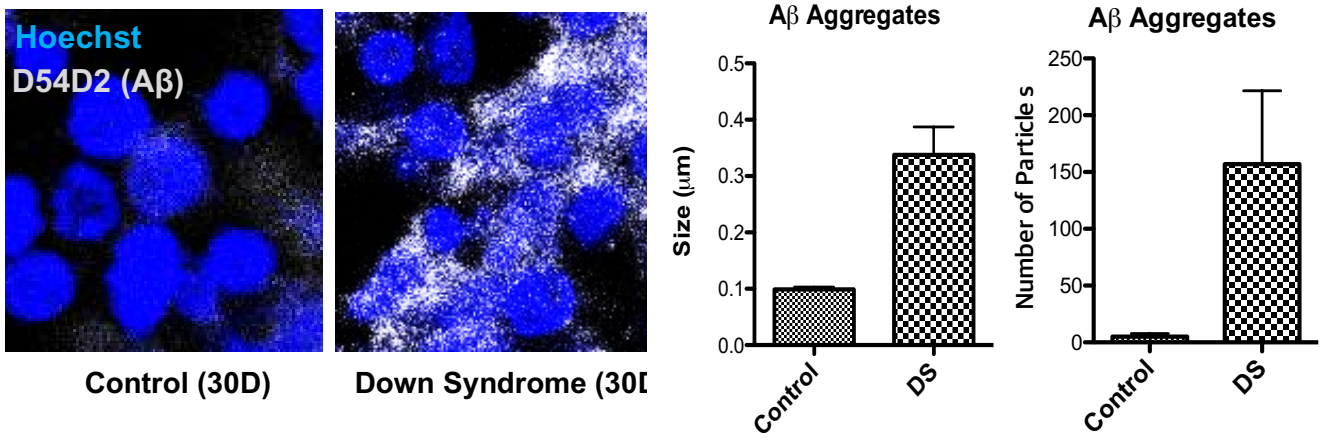
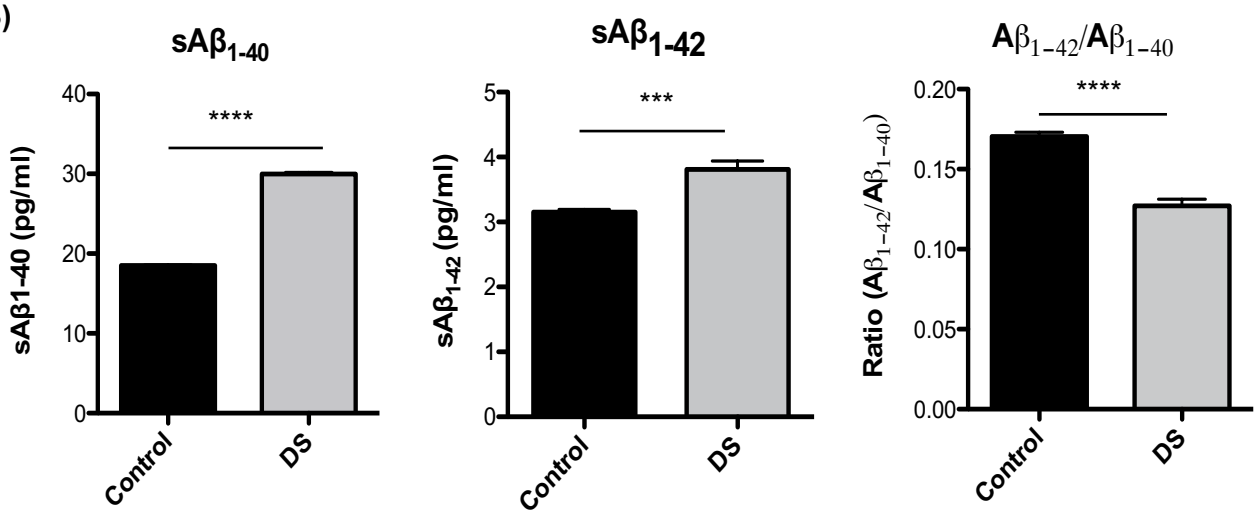


Figure 3.2 DS cerebral organoids have deficits in CNS development. A) The diameter of cerebral organoids was measured. DS cerebral oragnoids were smaller relative to the disomy 21 organoids. B) The pluripotency in each line was quantified by qPCR, showing that DS organoids had lower level of pluripotency both in OCT4 and in Nkx2.1. C) mRNA levels of genes for different brain regions were measured by qPCR and normalized to levels in DS.

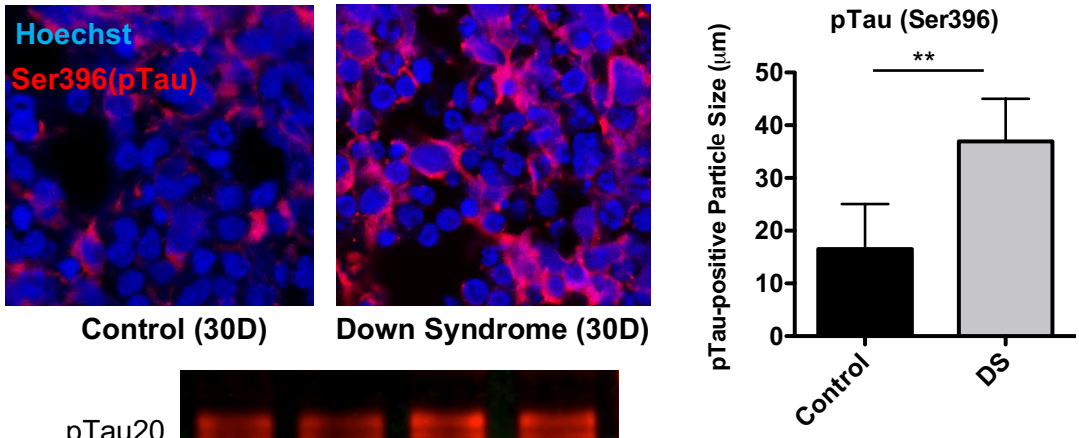
A)


Hoechst
D54D2 (Aβ)
Control (30D)
Down Syndrome (30D
Aβ Aggregates
Size (μm)
0.5
0.4
0.3
0.2
0.1
0.0
Control
DS
Aβ Aggregates
Number of Particle s
250
200
150
100
50
0
Control
DS

B)


sAβ1-40
sAβ1-40 (pg/ml)
40
30
20
10
0
****
Control
DS
sAβ1-42
sAβ1-42 (pg/ml)
5
4
3
2
1
0
***
Control
DS
Aβ1-42/Aβ1-40
Ratio (Aβ1-42/Aβ1-40)
0.20
0.15
0.10
0.05
0.00
****
Control
DS

C)


Hoechst
Ser396(pTau)
Control (30D)
Down Syndrome (30D)
pTau (Ser396)
pTau-positive Particle Size (μm)
50
40
30
20
10
0
**
Control
DS

D)

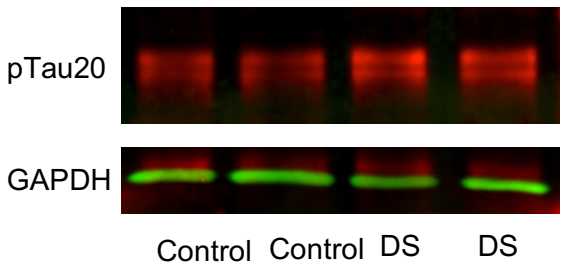
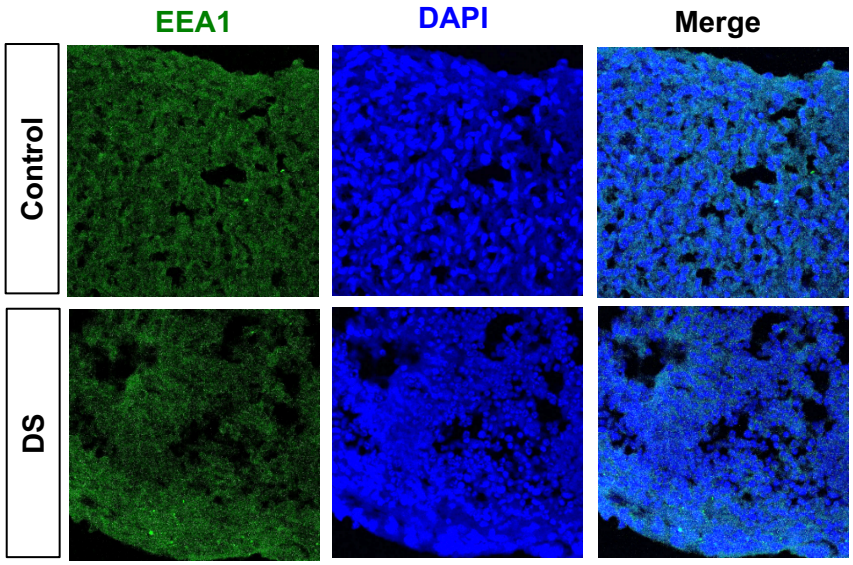
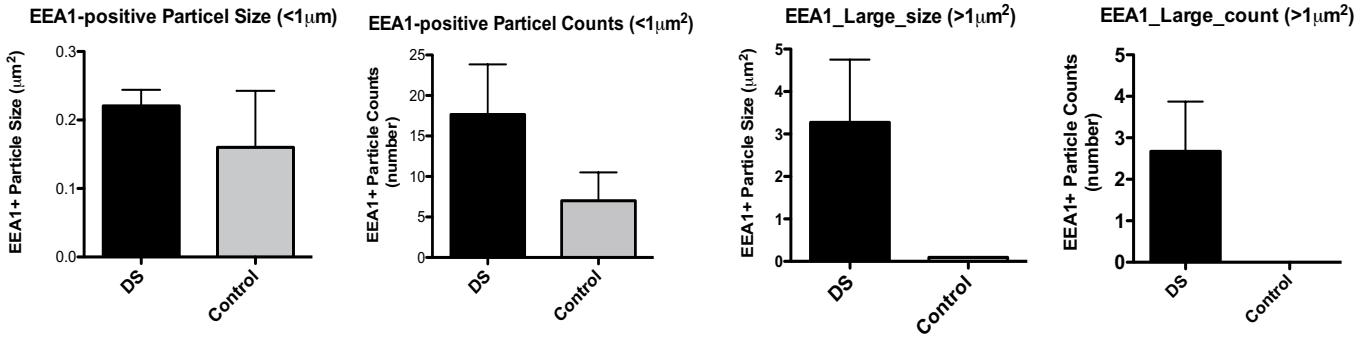

pTau20
GAPDH
Control
Control
DS
DS

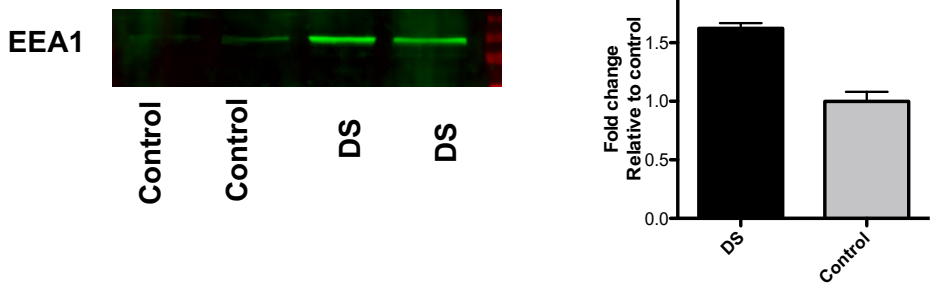
Figure 3.3 AD phenotypes could be recapitulated in DS cerebral organoids. A) Imunocytochemistry with antibodies to Aβ aggregates in DS (Left) and disomy 21 (Right) cerebral organoids. B) secreted levels of soluble Aβ42 and Aβ40 in culture media from cerebral organoids were measured by ELISA. C) immunocytochemistry with Ser396 pTau antibody in disomy 21 (Left) and in DS (Right) cerebral organoids. D) Immunoblotting with pTau20 antibody in lysates from the disomy 21 and the DS cerebral oragnoids.

E)


EEA1
DAPI
Merge
Control
DS


EEA1-positive Particel Size (<1μm)
EEA1+ Particle Size (μm²)
0.3
0.2
0.1
0.0
DS
Control
EEA1-positive Particel Counts (<1μm²)
EEA1+ Particle Counts (number)
25
20
15
10
5
0
DS
Control
EEA1_Large_size (>1μm²)
EEA1+ Particle Size (μm²)
5
4
3
2
1
0
DS
Control
EEA1_Large_count (>1μm²)
EEA1+ Particle Counts (number)
5
4
3
2
1
0
DS
Control

F)


EEA1
Control
Control
DS
DS
Early Endosomal Acitivities
Fold change Relative to control
2.0
1.5
1.0
0.5
0.0
DS
Control

G)

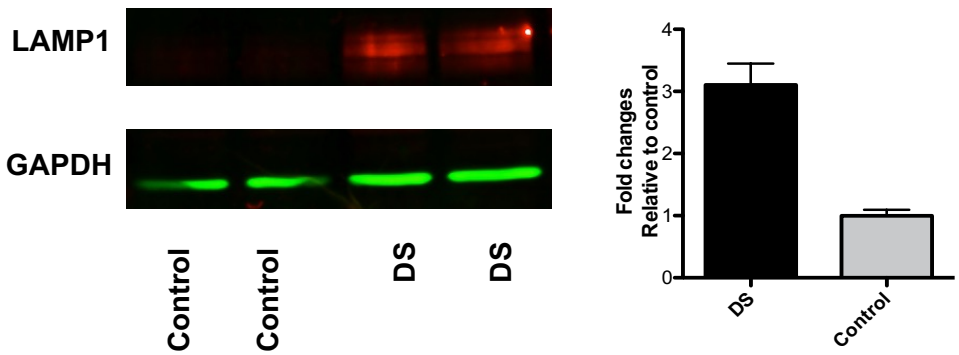
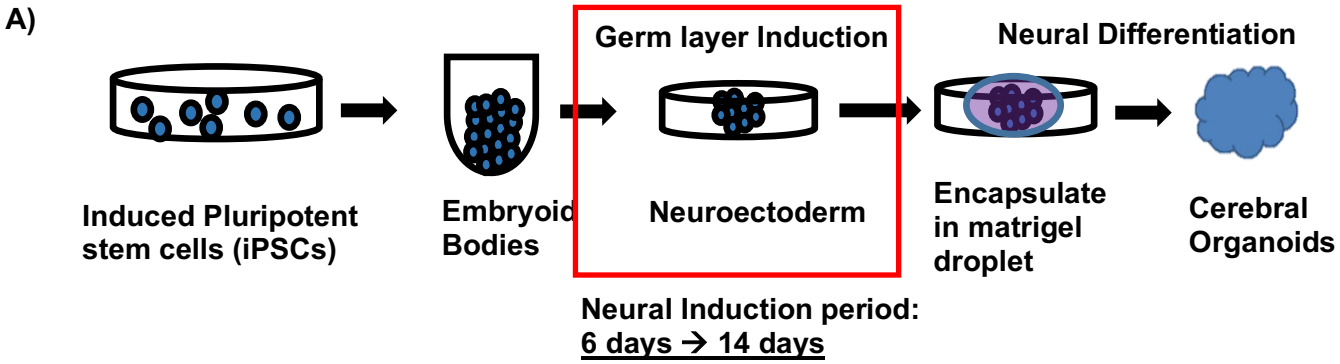
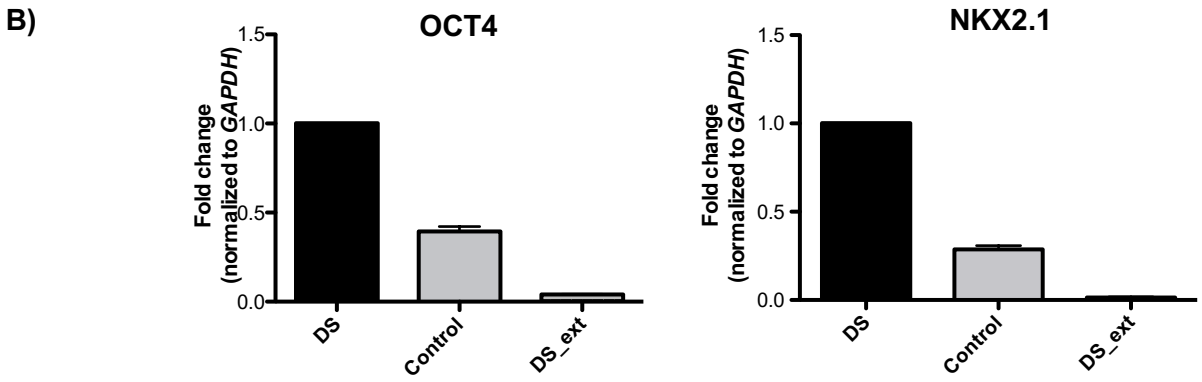

LAMP1
GAPDH
Control
Control
DS
DS
LAMP1 peptide
Fold changes Relative to control
4
3
2
1
0
DS
Control

Figure 3.3 (Cont'd) AD phenotypes could be recapitulated in DS cerebral organoids. D) immunocytochemistry with EEA1 antibody in disomy 21 (Upper) and in DS, trisomy 21 cerebral oragnoids (Bottom). Bar graphs represent the number and size of EEA1 particles in DS (black) and disomy 21 (grey) cerebral organoids. F) Immunoblotting with antibodies against EEA1 in lysates. Bar graphs represent relative immunoreactivity of EEA1 in each group normalized to the control. G) Immunoblotting with antibodies against LAMP1. Bar graphs represent the relative immunoreactivity of LAMP1 normalized to the control.


A)
Germ layer Induction
Neural Differentiation
Induced Pluripotent stem cells (iPSCs)
Embryoid Bodies
Neuroectoderm
Encapsulate in matrigel droplet
Cerebral Organoids
Neural Induction period:
6 days → 14 days


B)
OCT4
NKX2.1
Fold change (normalized to GAPDH)
DS
Control
DS_ext

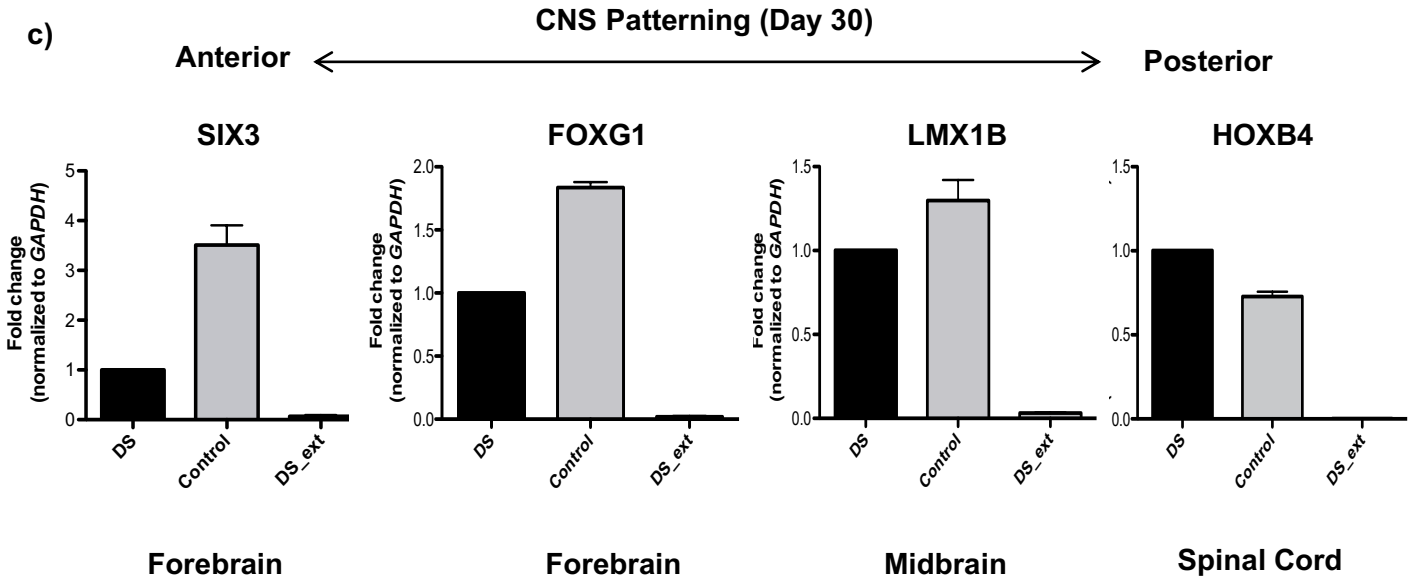
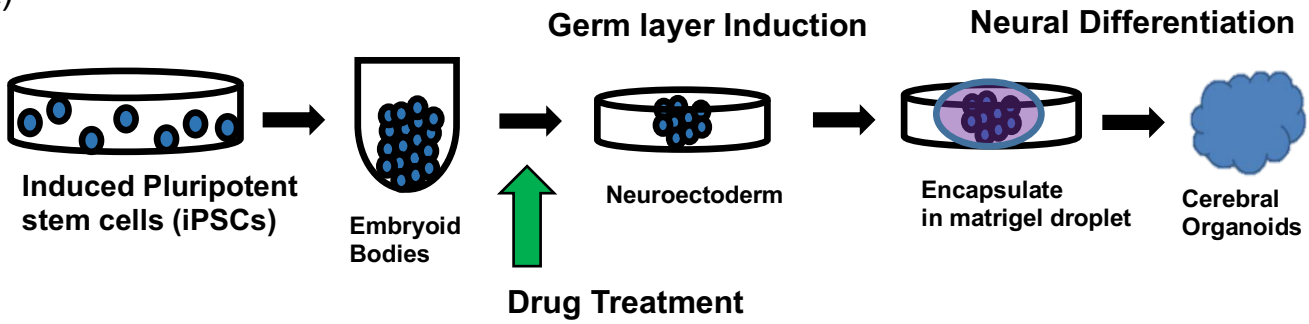
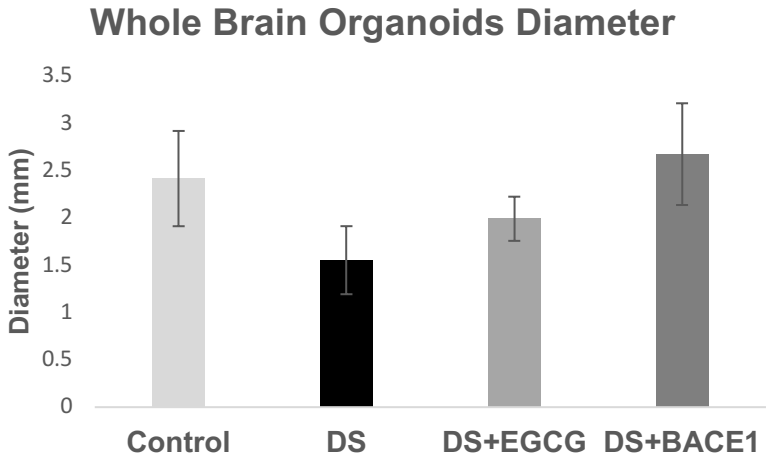

c)
CNS Patterning (Day 30)
Anterior
Posterior
SIX3
FOXG1
LMX1B
HOXB4
Fold change (normalized to GAPDH)
DS
Control
DS_ext
Forebrain
Forebrain
Midbrain
Spinal Cord

Figure 3.4 Developmental deficits could not be rescued by extending neural induction periods that facilitate proliferation of neural progenitor cells. A) A germ layer induction period was extended to 14 days, promoting developments in neuroectoderm marked in schematics. B) After extended periods of inducing neuroectoderm, cerebral organoids were derived following the regular procedures. Then pluripotencies on both disomy 21 and trisomy 21 cerebral organoids were measured by qPCR. C) CNS development patterns were measured by qPCR in both cerebral organoids. Each qPCR level was normalized to level in DS cerebral organoids.

A)


Germ layer Induction
Neural Differentiation
Induced Pluripotent stem cells (iPSCs)
Embryoid Bodies
Neuroectoderm
Encapsulate in matrigel droplet
Cerebral Organoids
Drug Treatment

B)


Whole Brain Organoids Diameter
Diameter (mm)
3.5
3
2.5
2
1.5
1
0.5
0
Control
DS
DS+EGCG
DS+BACE1

c)

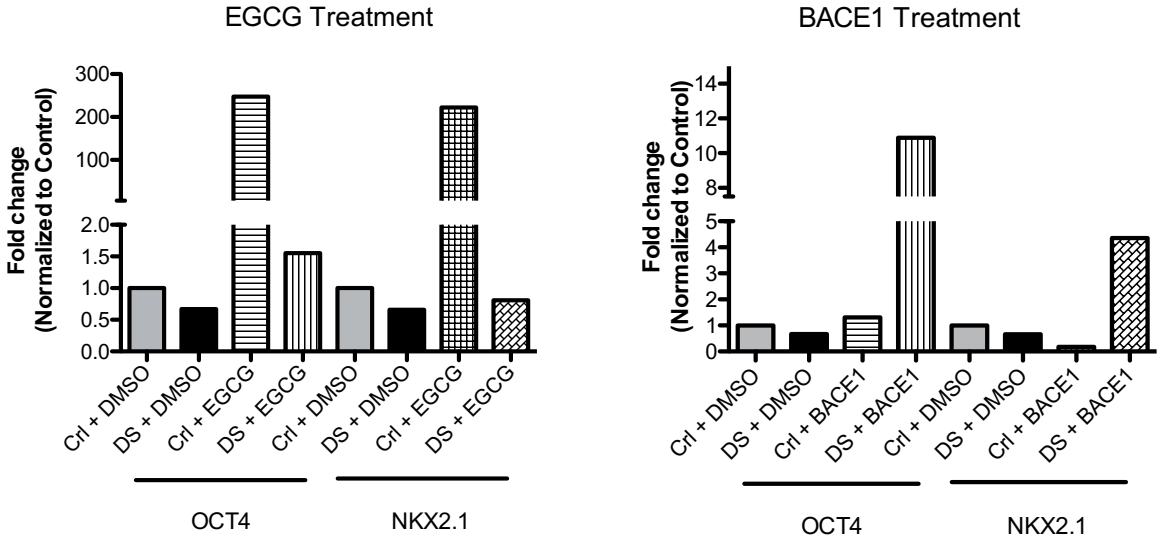
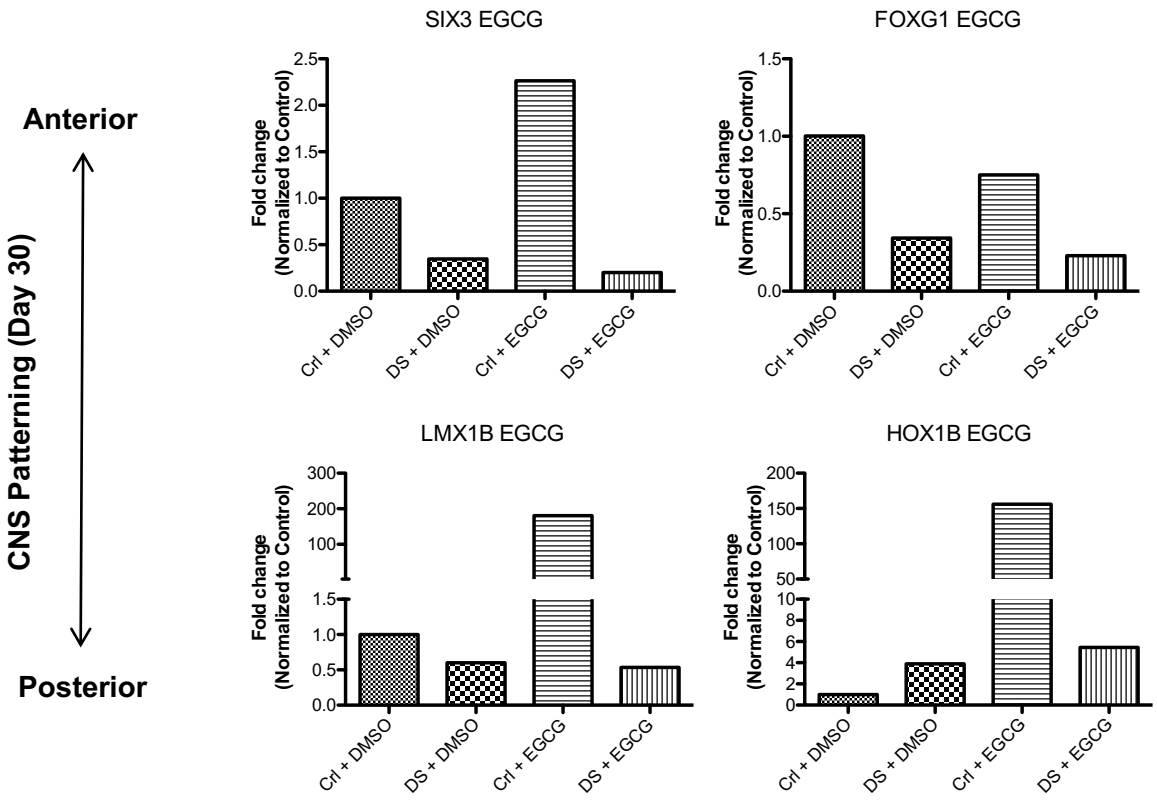

EGCG Treatment
BACE1 Treatment
Fold change (Normalized to Control)
Crl + DMSO
DS + DMSO
Crl + EGCG
DS + EGCG
Crl + BACE1
DS + BACE1
OCT4
NKX2.1

Figure 3.5 Developmental deficits could be alleviated by small compounds. A) Two different classes of drugs (EGCG and BACE1 inhibitor) were being administered from neuroectoderm induction period marked in the schematics and kept in the rest of procedures. B) Dimeters of organoids treated with each drug were measured. C) Measurement of the pluripotency in DS and disomy 21 oragnoids without and with drug treatments. EGCG(Left) and BACE-1 inhibitor (Right)

D)


Anterior
CNS Patterning (Day 30)
Posterior
SIX3 EGCG
FOXG1 EGCG
LMX1B EGCG
HOX1B EGCG
Fold change (Normalized to Control)
Crl + DMSO
DS + DMSO
Crl + EGCG
DS + EGCG

E)

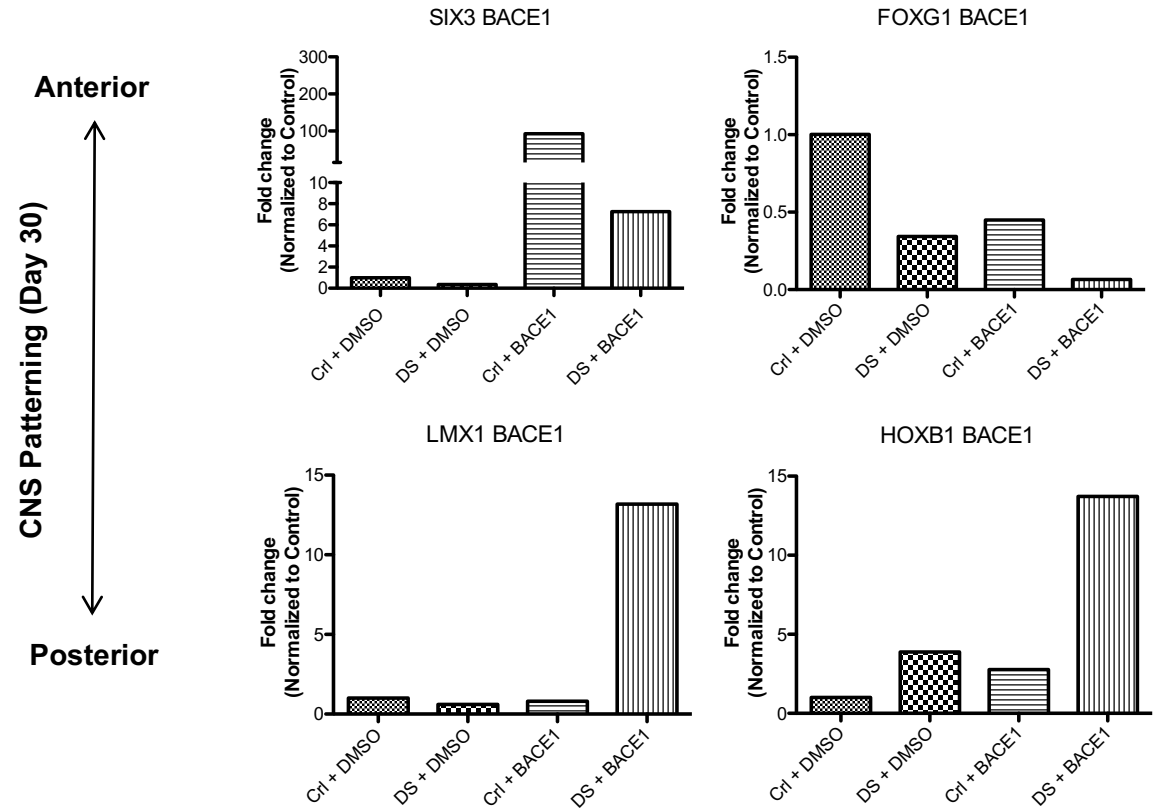

Anterior
CNS Patterning (Day 30)
Posterior
SIX3 BACE1
FOXG1 BACE1
LMX1 BACE1
HOXB1 BACE1
Fold change (Normalized to Control)
Crl + DMSO
DS + DMSO
Crl + BACE1
DS + BACE1

Figure 3.5 (Cont'd) Developmental deficits could be alleviated by small compound treatments. D) EGCG effects on developmental profile were measured by qPCR. Each measurement was normalized to the control treated with DMSO. E) BACE-1 inhibitor effects on developmental profile were measured by qPCR. Each level of qPCR measurement was normalized to the levels of disomy 21 cerebral oragnoids.

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Chapter 4: Implications and Future Direction

Introduction

This thesis has demonstrated the capability of modeling neurological diseases using induced pluripotent stem cells (iPSCs). In addition to the studies described herein, there have been many published reports modeling various neurological diseases[1,2,3–5]. These studies have proven the utility and its potentials of iPSCs technology for disease modeling and for developing therapeutic interventions. Despite these technological advances, there still are the rooms to improve for a better complete understanding of diseases. This chapter will discuss my opinions on how to address questions that arise from the previous sections.

Minimizing Unintended Inducible Variations

The recent advances in genome-editing technologies have addressed many concerns that arise from the results from the studies that did not have proper controls for inherent genetic variations of iPSC cell lines. Due to the ease and precise targeting of the CRISPR/Cas9 technology, it has been widely adapted and becomes beneficial for a broader range of experiments requiring for genome editing. On the other hand, these advanced technologies themselves due to their technical complication require for careful consideration over variations that these methodologies can induce. The first source arises from the genetic level. Despite the high fidelity of site-specific targeting by nucleases, unintended mutations have been reported to occur at off-target sites, indicating off-target possibilities[6]. Fortunately, patterns of inducing off-target mutations are not random. Thus those can be predicted by such algorithms offered from Harvard-MIT Broad institution[7].

Moreover, exome sequencing or whole genome sequencing experiments can be executed in two purposes: to screen out clone having off-target mutations and to examine any off-target mutations[8,9]. Another way to minimize the off-target effects is to determine whether the phenotypes of interest can be recapitulated in two or more independent clones generated by two different nucleases targeting sequences with little homology. Because each nuclease creates its off-target mutation profile, if the phenotype of interest is present, we can make sure that this phenotype has not been driven by off-target mutations.

The second source for variations also occurs at the genetic level: mutations during the experiments such as clonal isolation and maintenance of iPSCs. Recent whole-genome sequencing data have revealed that accumulation of spontaneous mutations (mostly, single nucleotide variant (SNV)) is not unavoidable in *in vitro* culture[10,11]. Since these mutations can occur in any proliferating cells via various mechanisms [11], a population of mitotically active iPSCs derived from fibroblast comprises a mixture of cells with random SNVs. Thus any cells isolated from the mixed population have a slightly different genetic background. Therefore, the best practice to minimize culture-induced genetic mutations is to utilize cell lines having lower passage numbers in any steps. Fortunately, the recent genome editing tools have been designed to minimize these mutations (for example, new CRISPR/Cas system enabling for multi-site targeting[12] and single-step targeting tool using oligos[13]).

Last source comes at the epigenetic level. Yeast studies have found that mammalian cells can produce epigenetically distinct progeny whose epigenome can

remain stably unchanged after passaging[14]. In principle, these epigenetic changes can occur between parental and gene-targeted cell lines, and this may pose a considerable problem for disease modeling if loci having epigenetic alterations are in charge of pathological phenotypes. Providentially, such technology as whole genome sequencing can conceive any altered epigenetic variability between lines. Furthermore, these identified alterations can be reset using epigenetic modifying small molecules. For instance, the DNA methyltransferase inhibitor 5-aza-cytidine (AZA) can remove methylation, converting to the basal methylation state[15]. In summary, careful considerations on multiple levels of variations should be counted in when designing experiments, and followed by careful selection over experimental methods in order not to mislead results and to maximize implications of findings.

Engineering three-dimensional tissues

Building three-dimensional brain tissues using cerebral organoids system[16], scaffold-based system[17] and neural spheroid[18] can provide more knowledge in depth in cellular compositions and dynamic cellular interactions of brain development including mature neuronal traits and functional neuronal networks. High-throughput single-cell transcriptional profiling method is one way[19]. A better understanding and utilization of these three-dimensional models will open limitless opportunity to explore and study various aspects of brain developments and organizations.

Identifying Pathological Pathways through RNA-Sequencing Data

In this thesis, I described two studies focusing on evaluating a risk factor or pathways for disease progression. While both studies successfully recapitulated multiple pathological phenotypes of corresponding diseases, providing insights for novel possible therapeutic targets, these studies further offer invaluable insights through transcriptional analysis. For example, transcriptional profiling on cells subjecting to any perturbation such as genetic mutation and small molecules of interest will display gene expression differences, highlighting specific gene(s) or pathway(s) relevant to disease progression[10].

How to Translate *in Vitro* Results to Clinical Trials

Although animals-based disease models provide many mechanistic insights on disease pathology, they have thus far not made much success in producing efficacious therapeutic interventions. This thesis has described the use of iPSCs to establish human neurological disease models, and surprisingly, identified the compound BACE-1 inhibitor as a potential modifier of DS pathologies. As shown in this thesis, iPSC-based models provide great hopes for identifying new drugs and of re-purposing available drugs. Its utility will be thoroughly examined via clinical trials using therapeutics discovered from iPSC models. In addition to this potential, this approach can alleviate cost-burdens on clinical trials and provide more effective manners for undertaking human trials. Instead of using animal models often compounding results from safety tests due to the inter-specifies difference, it is possible to in part replace the current safety test steps with human iPSCs model, in turn providing more precise and direct safety evaluations of drugs on a human.

Moreover, it can make the current design strategies for clinical trials more efficient and predictive. For example, instead of targeting the entire AD patients, re-stratifying patients into subgroups based on manifested *in vitro* phenotypes, and then testing drugs on those subgroups may reduce diluting effects of therapeutic benefits and increase the chance to discover more effective drugs for specific groups, which in turn facilitate drug-approval processes. Plus, this strategy can also be beneficial for the personalized medicine. Clinicians prescribe drugs only to patients' subgroups that have manifested phenotypes where drugs are just valid to rescue.

Conclusion

In this chapter, I have described my opinions for next-step studies in iPSC-based neurological disease modeling. Although there is a possibility that each area discussed herein plays a role against the promises of this model, each step can be a great avenue providing a better understanding of disease etiology and mechanistic insights. More careful experimental designs with considerations over diverse variation sources should be accompanied. The power of human stem cell-based models is that these can be utilized to discover therapeutic interventions as well as to replace current human clinical trials. In addition to clinical purposes, patient-derived stem cells can be used to find the most effective therapeutic methods for personalized medicine. In sum, these suggestions will contribute to improved understanding of neurological diseases and the best use of human iPSC-based models.

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