MODELING GENETIC RISK FACTOR OF ALZHEIMER'S DISEASE "*SORL1*" USING PATIENT SPECIFIC PLURIPOTENT STEM CELLS.

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medical Sciences
Common Course
FACULTY OF MEDICINE
Chulalongkorn University
Academic Year 2020
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การสร้างแบบจำลองของโรคอัลไซเมอร์ ที่มีความผิดปกติทางพันธุกรรมของ *SORL1* จากเซลล์ต้นกำเนิดจำเพาะ ชนิดพหุศักยภาพจากคนไข้

น.ส.กมลชนก คงศรี


วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์ ไม่สังกัดภาควิชา/เทียบเท่า
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ปีการศึกษา 2563


ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

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|---|---|
| Thesis Title | MODELING GENETIC RISK FACTOR OF ALZHEIMER’S DISEASE “*SORL1*” USING PATIENT SPECIFIC PLURIPOTENT STEM CELLS. |
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กมลชนก คงศรี : การสร้างแบบจำลองของโรคอัลไซเมอร์ ที่มีความผิดปกติทางพันธุกรรมของ *SORL1* จากเซลล์ต้นกำเนิดจำเพาะ ชนิดพหุศักยภาพจากคนไข้. ( MODELING GENETIC RISK FACTOR OF ALZHEIMER'S DISEASE "*SORL1*" USING PATIENT SPECIFIC PLURIPOTENT STEM CELLS.) อ.ที่ปรึกษาหลัก : รศ. ดร. นพ.นิพัญจน์ อิศรเสนา ณ อยุธยา

โรคอัลไซเมอร์เป็นโรคที่มีสภาวะเสื่อมทางสมองที่พบมากที่สุด และยังมีอัตราการเพิ่มขึ้นทุกปีในสังคมผู้สูงอายุ แต่อย่างไรก็ตามยังไม่มีการรักษาใดที่รักษาให้หายขาดได้ การหาแนวทางการรักษาใหม่จึงมีความจำเป็นเป็นอย่างมาก ในการศึกษาปัจจุบันพบว่ายีน *SORL1* นั้นเกี่ยวข้องกับการก่อให้เกิดความเสี่ยงในการเป็นโรคอัลไซเมอร์ชนิด sporadic เพิ่มขึ้นหลายเท่า ต่อมามีการศึกษาที่พบว่า *SORL1* นั้นช่วยลดปริมาณของอะไมลอยด์เบต้า โดยนำพาอะไมลอยด์เบต้าไปทำลายที่lysosome นอกจากนั้นยังพบว่าการเหนี่ยวนำให้เกิดการแสดงออกของ *SORL1* ที่มากกว่าปกติ สามารถทำให้ระดับของอะไมลอยด์เบต้าลดลงได้ด้วยเช่นกัน แต่อย่างไรก็ตามกลไกการแสดงออกที่เพิ่มขึ้นของยีน *SORL1* นั้นยังไม่ชัดเจน ในการศึกษานี้ ผู้วิจัยจึงได้สร้าง *SORL1-EGFP* reporter ในเซลล์ต้นกำเนิดชนิดพหุศักยภาพ (iPSC) ร่วมกับเทคนิค CRISPR/Cas9 เพื่อใช้ในการติดตามการแสดงออกของ *SORL1* จากผลการศึกษานี้แสดงให้เห็นว่าประสบความสำเร็จในการสร้าง *SORL1-EGFP* reporter iPSC cell lines ซึ่งมีการแก้ไขยีนได้อย่างถูกต้อง โดยยังคงมีคุณสมบัติพหุศักยภาพ และมีการแสดงออกในระยะของเซลล์ประสาทที่ถูกเหนี่ยวนำจาก iPSC นอกจากนี้ยังพบว่า *SORL1-EGFP* มีการแสดงออกในตำแหน่งที่ถูกต้อง บริเวณ early endosome และ lysosome lines ตามที่มีรายงานมาก่อน และมีการแสดงออกของ *EGFP* ที่เพิ่มมากขึ้นหลังจากที่ถูกเหนี่ยวนำด้วยตัวกระตุ้น BDNF และ cAMP ดังนั้น *SORL1-EGFP* reporter iPSC-derived cortical neurons สามารถติดตาม *SORL1* ในเซลล์ประสาทได้อย่างถูกต้องและแม่นยำ อีกทั้งงานวิจัยนี้ยังมีการศึกษาการกระตุ้นการแสดงออกของ *SORL1* ด้วยเทคนิค CRISPR-on ซึ่งเป็นเทคนิคที่มีความจำเพาะต่อendogenous ของยีนสูง จากผลการศึกษาพบว่า sgRNASORL1 นั้นสามารถกระตุ้นการแสดงออกของ *SORL1* ใน human erythroleukemic cell line (K562) เพิ่มขึ้นอย่างมีนัยสำคัญ จากผลการศึกษานี้อาจนำไปใช้เป็นเครื่องมือในการศึกษาการทำงานของ *SORL1* เพื่อนำไปให้ทดสอบการหายาชนิดใหม่ในการรักษา รวมทั้ง sgRNASORL1 ที่ได้นี้อาจมีประสิทธิภาพเพียงพอที่จะสามารถนำไปกระตุ้นการแสดงออกของ *SORL1* ในเซลล์ประสาทของผู้ป่วยโรคอัลไซเมอร์ เพื่อเป็นแนวทางการรักษาใหม่ของโรคอัลไซเมอร์ต่อไปในอนาคต

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# # 5974050830 : MAJOR MEDICAL SCIENCES

KEYWORD: Alzheimer's Disease (AD), Sorting-related receptor with A-type repeats (SORL1), Induced pluripotent stem cell (iPSC), CRISPR/Cas9, SORL1-EGFP iPSC reporter line


Kamonchanok Kongsri : MODELING GENETIC RISK FACTOR OF ALZHEIMER'S DISEASE "*SORL1*" USING PATIENT SPECIFIC PLURIPOTENT STEM CELLS.. Advisor: Assoc. Prof. NIPAN ISRASENA, M.D., Ph.D.


Alzheimer's disease is the most common neurodegenerative; cause of dementia and trends to increase in elder society. There is still not cure. A new strategy development for treatment is needed. *SORL1* is a major genetic risk factor associated with sporadic AD. It correlates with degrading amyloid beta (Aβ) by sorting to lysosome. *SORL1* overexpression has been reported that reduce Aβ production. However, the molecular mechanism that regulate in neurons is still unclear. The purpose of this study is to generate an effective drug discovery platform for identifying molecules that can modulate *SORL1* level in human neurons by using induced pluripotent stem cells (iPSCs) together with genome editing using CRISPR/Cas9 technology to generate human cortical neurons *SORL1-EGFP* reporter cells. Here, we demonstrated that we successfully generated *SORL1-EGFP* reporter iPSC cell lines which still retains pluripotency and when differentiates to cortical neurons *in vitro*, express *EGFP* at the location corresponding to the location of endogenous *SORL1* and *SORL1-EGFP* could more expressed when induced by BDNF and cAMP. Moreover, we generated the activation *SORL1* using CRISPR-on technique which highly specific to endogenous. The results indicated that sgRNASORL1 could activate and significantly increase *SORL1* expression levels in human erythroleukemic cell line (K562). This model could advance our understanding of *SORL1* regulation, mechanisms of neurodegeneration and the effective of *SORL1* activated sgRNASORL1 might offer a platform to find new therapeutics for Alzheimer's disease in further.


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| Field of Study: | Medical Sciences | Student's Signature ............................... |
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| Academic Year: | 2020 | Advisor's Signature ............................. |

# ACKNOWLEDGEMENTS

This thesis would not have been possible without the support and nurturing of many people. First, I would like to express the deepest appreciation to my advisor, Assoc. Prof. Nipan. Israsena Na-Ayudhya, M.D., Ph.D. for valuable suggestions, encouragement and supportive guidance. My deepest appreciation goes to Dr. Praewphan Ingrungruanglert for her kindness, training, advice and always being understanding thought the course of my study. And I would like to express my deepest appreciation to my committee including Assoc. Prof. Poonlarp Cheepsunthorn, Ph.D., Assoc. Prof. Dr. Wilai Anomasiri, Ph.D., Dr. Supansa Yodmuang, Ph.D. and Dr. Ruttachuk Rungsiwiwat, Ph.D. for their comments and suggestions.

I would like to thank Natthida Kittimawikrom, my lab buddy, for being listener and all helps me along with my master's degree.

I would like to thank Sarinya Poadang for her kindness about cell cultures and thanks to Netchanok Leela-adisorn for flow cytometry analysis and CRISPR technique.

Thanks for my lovely friends in the Stem cells and cell therapy research unit, King Chulalongkorn Memorial hospital and Chulalongkorn University that being supporting other ways they could.

Finally, I would also like to express my gratitude to my family for their financial support and cheered me up whenever I had rough time. Thanks for Sean Xiao and Suparada Khanaruksombat for remedy my suffering and being my inspiration.

Kamonchanok Kongsri

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# CHAPTER I

# INTRODUCTION

## 1. Background and Rational

Alzheimer's disease (AD) is the most common neurodegenerative disorder, characterized by progressive loss of memory and cognitive function. This damage effect to their daily life. Worldwide, patients who live with AD are estimated about 50 million and predicted to increase to 130 million in 2050 [1]. The pathologies of this disease are generally having brain atrophy and amyloid-β (Aβ) deposited extracellular while neurofibrillary tangles (NFTs), composed of hyperphosphorylated tau intracellular which observed when stain with silver stain in the brain. The current treatments for AD target cholinergic and/or glutamatergic neuronal function, providing cognitive function, but some patients do not respond. Moreover, there are no effective cure for AD.

The AD is classified into 2 types; Early onset AD (EOAD), familial AD (FAD) and late onset AD (LOAD), sporadic AD (SAD). The FAD mainly caused by the mutation in three genes coding for the amyloid precursor (APP), the presenilins1 and 2 (*PSEN1* and *PSEN2*) that play a role in amyloid processing, strongly support the amyloid cascade hypothesis in AD [2, 3]. In contrast to SAD are poor understood genetic risk factors and the underlying molecular and cellular mechanism of SAD is largely unknown. The strongest risk factor for SAD was first identified in 1992 is Apolipoprotein E4 (APOE4) [4]. Heterozygous of APOE4 (E3/E4) can increase AD risk by 3-5 times while homologous (E4/E4) can increase the risk by 10-15 times. Apart from APOE4, more than 29 genetic risk factors strongly associated with AD have been identified by using candidate gene approach and genome-wide association study [5].

The sorting protein-related receptor (*SORL1*) was reported as another severe risk factor. Reduction of SORL1 expression level is associated with AD. The effect of SORL1 on AD risk ranged from small modifying effects to causative effects depending on its variants. Some variants are associated with 12-folds increased in the risk, comparable to that carrying heterozygous APOE4 [6]. Interestingly, despite the

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pathogenic effect, *SORL1* plays a protective role against Aβ neuronal secretion, by binding to the amyloid precursor protein (APP), preventing its processing into Aβ or directing it to the lysosome for degradation [7]. Overexpression of *SORL1* reduced Aβ processing both *in vivo* [8] and *in vitro* [9]. In contrast, lower expression of *SORL1* could increase Aβ production and senile plaque in mice. As mention previously, the *SORL1* can prevent the Aβ toxicities in brain. Nevertheless, the molecular pathway that regulates *SORL1* function in neurons is still unclear.

Development of new AD treatment is limited by the lack of reliable AD model. Mouse model, however, could not develop the disease as it develop in human [1, 10]. Neural cell derived from patient brain is an ideal screening system. In this study iPSCs knocked in *SORL1-EGFP* were successfully generated using CRISPR Cas9 to modulate *SORL1* expression in neural cells. Moreover, CRISPR-on system was designed for *SORL1* upregulation. The sgRNAs of CRISPR-on system were success and efficiency to activate the *SORL1* transcription regulatory site in K562 cells. The most efficiency activation was the combination of sgRNASORL1-1,3,4 which upregulated *SORL1* expression to 600-fold. This model could advance our understanding of *SORL1* regulation, mechanisms of neurodegeneration and the effective of *SORL1* activated sgRNAs might offer a platform to find new therapeutics for Alzheimer’s disease by combine RNP technology with nanoparticle delivery system that can bring RNP pass through blood brain barrier into brain and function in mouse model [11] in further.

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# CHAPER II

# LITERATURE REVIEWS

## 2.1 Alzheimer’s disease

Alzheimer’s disease (AD) is the most common neurodegenerative disorder. It is mostly presence in elderly people with dementia. This damage interferes with memory loss, thinking skills and language abnormalities. The AD has become one of important public health problems and disturbs patients’ daily life and their family to look after. Worldwide, patients who live with Alzheimer’s disease are estimated about 50 million and predicted to upsurge to 130 million in 2050 [12]. The estimated global cost of Alzheimer’s disease in 2018 is 1 trillion dollars US and will be rising to 2 trillion dollars by 2030 [12, 13]. The pathologies of Alzheimer’s disease were first examined on Auguste D.’s brain by Alois Alzheimer in 1906. The results showed that the patient brain had a widespread on an anatomical level and on a histological level. The brain tissue was stained with Bielschowsky’s silver [14]. The finding presented the peculiar thick and strongly staining fibrils which clump into tangles and the enrichment of miliary foci which those plaques throughout the cerebral cortex. Afterwards, those pathologies have been known as amyloid plaques; amyloid beta accumulation and neurofibrillary tangles; accumulating of short segments of the microtubule-associated protein tau[3]. Those become the important hallmarks of AD. Alzheimer’s disease is classified to 2 types; Early onset AD (EOAD) and Late onset AD (LOAD). The EOAD or Familial AD (FAD) with 5% of AD cases. The patient with FAD possibly has at least 2 family members with AD. The AD almost presents before age 65. The cause of FAD has been reported as the mutation of *presenilin1 (PSEN1)*, *presenilin2 (PSEN2)* or amyloid precursor protein (APP) which are associated with amyloid processing. In the other hand, the LOAD or Sporadic AD with 95% of cases. It presents in a patient who is less than 65 years old. The cause of SAD is still not identified.

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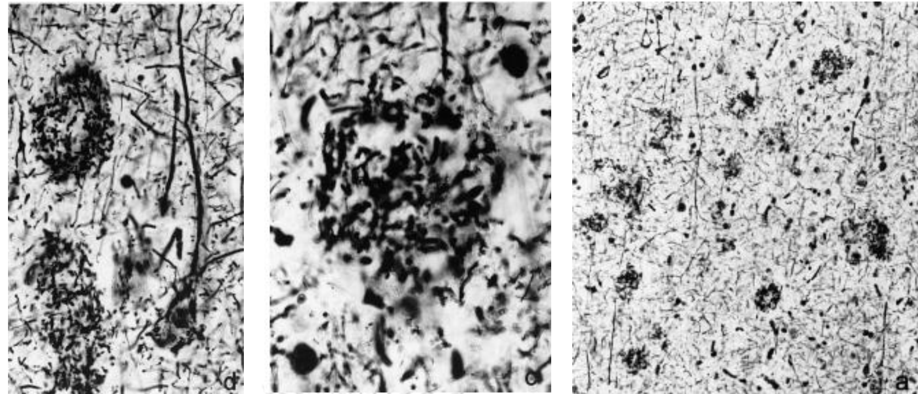

Figure1 Bielschowsky-stained tissue sections. Numerous amyloid plaques and neurofibrillary tangles are visible in the cerebral cortex of Auguste D. (M.B. Graeber., Histopathology and APOE genotype of the first Alzheimer disease patient, Auguste D.)

## 2.2 Amyloid-beta (Aβ)

Several studies base on mechanism that found in FAD. Processing of Aβ takes place by two pathways; amyloidogenic pathway and nonamyloidogenic pathway. On one hand, nonamyloidogenic pathway produces non-toxic soluble fragments via cleavages APP by alpha and gamma secretase named sAPPα. In the other hand, amyloidogenic pathway generates the toxic insoluble Aβ42 fragment via cleavages APP by beta and gamma secretase named Aβ and very common encounter in AD patients. From the FAD studies indicated that APP mutation increases beta secretase cleavage and *PSEN1, PSEN2* mutation results in cleavage site of gamma secretase on APP. The aberrant processing of the Aβ protein precursor induces the amyloid plaques though amyloidogenic pathway by induces Aβ accumulation. This pathology is the most toxic and leading to neuronal dysfunction. The Aβ peptide is classified into three groups followed length, molecular weight and microscopic dimensions criteria: (i) very short oligomers, (ii) small oligomers (17-42kDA) which Aβ–derived diffusible ligands (ADDLs) and (iii) protofibrils which could detect during mature amyloid fibrils formation *in vitro*.

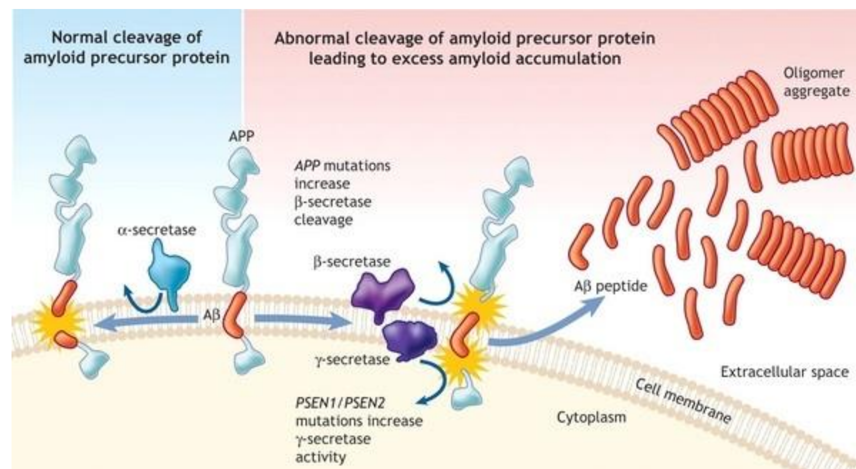


Figure 2 APP processing (Amyloid Protein Transmission through Neurosurgery, available: http://debuglies.com/2018/02/17/amyloid-protein-transmission-through-neurosurgery/)

Those are potentially neurotoxic and may cause neurotoxicity in AD. Two main toxics of Aβ are Aβ40 and Aβ42 which can form Aβ fibrils. The Aβ42 is reported that it is more toxicity than Aβ40. Most reports reveal that the extracellular deposits of Aβ in AD brain. However, Aβ have been reported that it begins in intracellular [15]. Reduction of Aβ has been a target for AD therapy by the knowledge about the mechanism. The beta-secretase (BACE1) inhibitor has been studied for AD drugs. It demonstrated that high doses of BACE1 inhibitor not only reduce Aβ plaques, but it also causes the toxicities for neurons. Thus, it may take more serious adverse events in elderly AD patients. The concern about limitation of using BACE1 inhibitor has been considered about the concentration of usage dose [6, 16]. Although, numerous drugs have been developed for AD therapies, it still has toxicity and side effects.

## 2.3 Intracellular Amyloid-beta

The Aβ was first determined as a constituent of extracellular amyloid. Afterwards, intracellular amyloid has begun to report to present in the literature [17]. The first study established that intracellular Aβ was stained with Aβ antibody and was observed in neurons in the cerebellum, cerebrum and spinal cord. The existence of Aβ might not be dependent on ages. Moreover, the result from Aβ staining was

observed in NFT. Thus, Aβ and tau pathology might be associated as the Aβ may promote tau pathology by triggering specific kinases [18]. In the immunogold electron microscopy presented the Aβ42 can be established in multivesicular bodies (MVBs) of neurons in normal human's brain. Furthermore, the results from immunoelectron microscopy indicated that intracellular Aβ42 was increased by aging in transgenic mice's and human's brain with Alzheimer's disease. It accrued in MVBs within presynaptic and postsynaptic compartments. Therefore, intracellular Aβ deposit was associated with abnormal synaptic morphology before Aβ plaques [19] and it may be an early event in the pathogenesis of AD [20]. Inhibiting intracellular Aβ accumulation may be an essential for AD therapy [20]. In 1995, intracellular Aβ was formed in Swedish mutant APP while wild-type APP did not[21]. It was similar to the reported in 2006 that human APP gene is related with upper levels of intracellular Aβ [22]. Likewise, evidence established that intracellular Aβ is produced in secretory pathway [23]. Retention of APP in the ER has been reported that blocks Aβ40 production whereas Aβ42 can be produced in the trans-Golgi network [24]. The extracellular deposits of Aβ, it is occurred in intracellular before secreted and it is taken up into the cells through receptor or transporters. The Aβ is generated as a monomer and gladly groups to form multimeric complex as protofibrils and fibrils. In human brain, Aβ oligomers formation initiates within cells rather than in extracellular space [25]. The intracellular was abundantly present but did not correlate with plaque or NFT formation. The finding does not mean that intracellular Aβ in AD does not exert pathological effects through other pathway as synaptic degeneration. The Aβ accumulation within MVBs is leading to disrupted MVB sorting via interruption of proteasome system and led to the buildup of tau protein in transgenic mouse model [26]. Furthermore, the intracellular Aβ in mitochondria is correlated with diminished enzymatic activity of respiratory chain complexes III, IV and a reduce rate of oxygen consumption [27]. There is evidence for a role for intraneuronal Aβ synaptic dysfunction. Numerous of factors have been indicated in animal models of AD. The interesting observation is the effect of aging. The early AD patient brain might have more enrich intraneuronal Aβ, which becomes extracellular and neuronal death in the next step. Other environmental and pharmacological factors can modulate the intracellular Aβ. Several studies indicated

that dietary treatment in mouse model reduces the soluble Aβ and intracellular. The intracellular and soluble Aβ increased but not reduced extracellular and oligomeric Aβ with the net effect being improved cognition. Therefore, the decreasing in extracellular was highly advantage, although intracellular Aβ increased in age mice with established extracellular Aβ pathology. In addition, other factors have been presented to increase the intracellular Aβ and are also associated with increased risk of AD. These include stress hormones, increased dietary cholesterol, oxidative stress, homocysteic acid and presence of ApoE-ε4. Numerous evidences from mouse models showed that intracellular Aβ can disrupt synaptic activity and lead to proteasome dysfunction, cause calcium dyshomeostasis and even facilitate Tau hyperphosphorylation. However, in animal model evidence of intracellular Aβ have been determined, evidence in human AD is required.

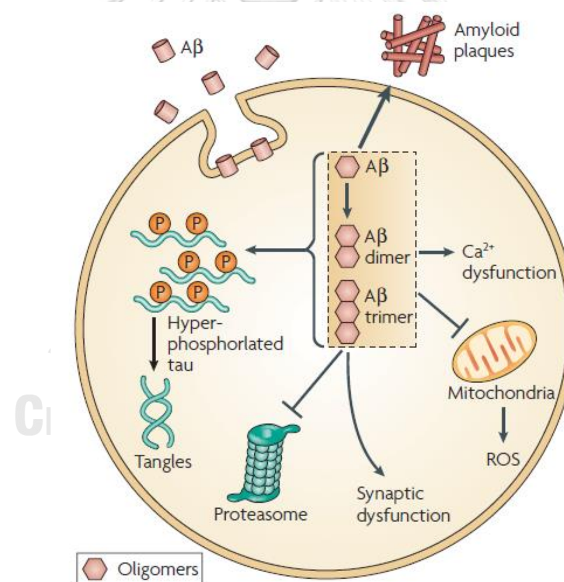


Figure 3 Pathological effects of intraneuronal Aβ (Frank M., *et al.,* Intracellular amyloid-β in Alzheimer's disease, Nature Reviews, Neuroscience vol. 8, July 2007.)

**2.4 Induced Pluripotent stem cell technology**

Animal model were used for study mechanism and drug tests, but animal do not represent the pathologies of AD as it happens in humans. Thus, we aim to find a new model that can develop the disease as it develops in humans. One of them is induced pluripotent stem cells (iPSC). After Yamanaka and teams achieved the cells reprogramming via transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) which iPSCs have the properties as embryonic stem cells, self-renew and can differentiate into all cell types of the body. Moreover, iPSCs can carry the genetic background as patients' cells. Human iPSC models have been generated for tool study including AD modeling. Human iPSC-derived neuron is a useful tool for studying the normal function and those cells are similar to original cell types. The patient cells were differentiated to neurons. The results found that cells could remain genetical mutation in patient. It is convenient to study and follow up. So that is why iPSC has become the effective tool to generate the Alzheimer's disease model. First things to do after generating iPSCs-derived neurons, it has to check the pathologies that present in the model such as amyloid oligomer and neurofibrillary tangle in a short time period. At the beginning FAD was used for the studies models since it has more severe manifestation than SAD. Several studies established that FAD-iPSCs-derived neurons produced higher levels of amyloid and tau phosphorylation. In addition, they can use for drug tests. For example, in 2013, Kondo *et al*., indicated that Aβ oligomers accumulated in iPSC derived neurons and astrocytes from patient cells with a familial amyloid precursor protein (APP)-E693D mutation and sporadic AD, leading to endoplasmic reticulum (ER) and oxidative stress. And docosahexaenoic acid (DHA) treatment alleviated the stress responses in the AD neural cells [10]. Form these results, AD-iPSC-derived neurons could show the mechanism and can use for drug test which appropriate in each subtype. Furthermore, most risk factors of SAD have been studied as association studies and genome-wild association study (GWAS). The first identify risk factor of SAD is *APOE4* in 1992 [4].

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## 2.5 Apolipoprotein E (ApoE)

Apolipoprotein E is located on chromosome 19. It is a major of apolipoprotein and a cholesterol carrier in the brain. It is 34kDa protein which transports cholesterol and other lipids in the plasma and central nervous system (CNS). The APOE has three different polymorphic alleles: ε2, ε3 and ε4. ApoE- ε2 is associated with protection late onset AD compare with ε3. And the ApoE- ε4 is recognized as the strongest genetic risk factor for AD. The frequency of ε4 is about 15% in general populations but about 40% in AD patients. Heterozygous of ε4 (ε3/ ε4) can increase risk by 3 to 5 times while homologous of ε4/ε4 can increase the risk by 10-15 times [28]. Furthermore, APOE receptors play important parts in both production and clearance processes. It interacts with APP and transits its trafficking and processing to Aβ. Low density lipoprotein receptor (LDLR) related protein (LRP1) binds to soluble APP which contains the Kunitz-type protease inhibitor (KPI) domains extracellularly. Thus, LRP1 and APP interact as enhanced APP endocytosis trafficking and processing to Aβ. However, the precise mechanisms through which *APOE4* contributes to enhanced AD risk remains unclear. And not only *APOE4* can be a risk factor for SAD, but also *SORL1* is associated with SAD [29].

## 2.6 Sortilin-related receptor1 (SORL1) in AD

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*SORL1* has been demonstrated that is one of genetics which is associated with AD. The AD patient brains presented the lower expression of *SORL1* and increased Aβ accumulation. This gene belongs to two families: low density lipoprotein receptor (LDLR) family and vascular protein sorting 10 (VPS10) domain receptor family. It is located on chromosome11 and contains the domains as NH2-terminal, a segment homologous to VPS10, five tandem LDLR 'YWTD' repeats, a cluster of 11 complement-type repeats (CR), the fibronectin-type (FN) III, a putatative membrane spanning region and COOH terminus. The *SORL1* contains numerous proteins called a hybrid receptor with multifunction including cargo transport, chaperone-like activities, signaling and intracellular sorting. The *SORL1* can bind the apolipoprotein E (ApoE) which is associated with AD and it can also bind VSP10 which is associated with amyloid beta

precursor (APP) in FAD that transfer APP from cell membrane to Golgi and endoplasmic reticulum [6]. The mutation of *SORL1* is established rare but it is associated with a five-fold increased risk for early onset AD, comparable to that of carrying the $\varepsilon 4$ allele of *APOE* [5]. As in 2007, the study indicated that the difference patterns of SNPs in *SORL1* were found that they associated with increase or decrease risk of AD patients even it is on same SNPs. It also depends on their race and genetic background. For example at the 5’ end of *SORL1*, CGC haplotype at SNPs 8-10 showed increased risk for AD in Caribbean Hispanic, Israeli Arab and North European data sets whereas TAT haplotype on same SNPs showed reduce risk in those data set or TTC haplotype at SNPs 19, 23-25 presented increased risk in North European but ACC haplotype on same SNPs showed as protective SNPs to decrease risk for AD in African-American data set and have risk in AD in Asian population[7]. Moreover, recently study indicated that the lower in *sortilin-related receptor1 (SORL1)* expression can increase the levels of Aβ but overexpression of *SORL1* could decrease it [10]. There are supported studies in hiPSC-derived neurons with *SORL1* mutation. The results presented that two variants of *SORL1* are associated with SAD: R as risk and P as protective variants. After the hiPSC-derived neurons with *SORL1* mutation were challenged with brain-derived neurotrophic factor (BDNF) and cyclic AMP (cAMP), respectively. The levels of *SORL1* expression were increased and the levels of Aβ were reduced in neurons which have P variant in *SORL1* after treating BDNF. While reduction of Aβ was not significantly differences between neurons with P or R alleles after treating cAMP. Thus, the study suggested that decreasing of Aβ in APP processing via treating BDNF was dependent on *SORL1* whereas cAMP may reduce Aβ in additional pathways to *SORL1*. Moreover, the study presented that *SORL1* overexpression could decrease the levels of Aβ including the case that BDNF could not do[9]. However, it unclears which specific variants of *(SORL1)* are keys risk factors for AD. The understanding about *SORL1* can bind APP and affect its trafficking and processing was indicated in cultured cells and in the mouse brain in vivo cells from Andersen OM et al. research in 2005. In 2006, Offe K. and teams showed that SORL1 overexpression significantly changed the levels of full-length APP (reduced 30%), C-terminal APP fragments (CTFs) (increased 2.5-fold) and extracellular Aβ (reduced 50%). Moreover, they found that both SORL1 and APP localized to the

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endosomal system. To our knowledge, a main APP trafficking and processing is the most striking SORL1 role to decease the levels of Aβ in AD. First, SORL1 binds APP to exist it in the TGN and prevents APP to form Aβ fragments from β-secretase (BACE1). And secondary, SORL1 binds Aβ to direct its to the lysosome [7]. Even if the SORL1 mechanism in APP trafficking and procession is still poor understood.

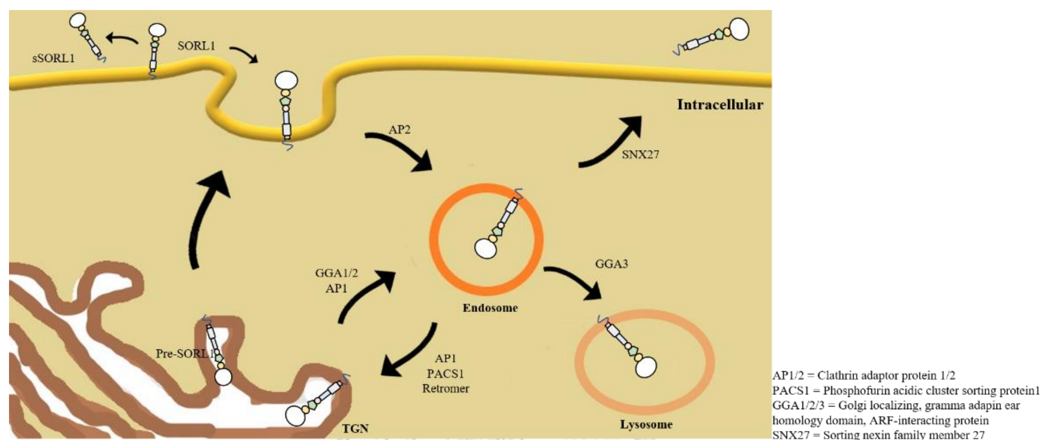


Figure 4 Intracellular trafficking path for SORL1. A nascent SORL1 is an inactive pro-receptor. It is activated by convertases in TGN which removes an amino-terminal pro-peptide and secretes to the cell surface through secretory pathway. Some molecules of SORL1 release of the extracellular in a soluble termed (sSORL1). Most SORL1 molecules at the cell surface undergo clathrin-dependent endocytosis facilitated by AP2. The vesicle moves from endosome back to the TGN to continuously shuttle between TGN and endosome afterwards. The anterograde movement of SORL1 from the TGN to endosomes is guided by GGA1 and GGA2, whereas the retrograde from endosomes back to the TGN is sorted by PACS1and retromer. AP1 may be involved in bi-directing sorting. Moreover, the alternative routes, SNX27 may sort SORL from endosome to the cell surface or to lysosomes by GGA3. Figure adapted from Andersen OM. et al.,2016

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## 2.7 CRISPR-ON system

Homologous recombination is a classical method for gene modification, but it takes more than a year. So new gene editing technology has come up instead and they have more abilities and more efficiency [30] such as Zinc finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs) and the newest tool, Clustered

regularly interspaced short palindromic repeats (CRISPR). Cas protein is a CRISPR-associated and utilized by bacteria and archaea to defend against viral pathogens. Cas 9 can bind the specific genomic locus or multiple loci via guided by the engineered guide RNA (gRNA). CRISPR-ON is one type of CRISPR tools which used dCas9. The dCas9 is a mutation of Cas9 without endonuclease activity and work with transcriptional activators and gRNA. dCas9 will bind the specific site on locus though navigator of gRNA and transcriptional activators (VPR) will activate the targeted gene [31]. Recently, Inoue et al. indicated that APP and BACE1 in normal and FAD fibroblast derived neurons were increased to 2.6-fold and 30.9-fold, respectively by SAM-mediated transcriptional activation which is one of technics in CRISPR on system. Activation of APP but not BACE1 also showed that the levels of amyloid were increased. Moreover, they compared the SAM activation with typical lentiviral overexpression of APP or BACE1. The result showed that the levels of APP or BACE1 expression by lentiviral overexpression was higher than SAM technic but performed to be toxic to the fibroblast [32]. So, CRISPR-on system can activate gene efficiency.

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# CHAPTER III

# RESEARCH METHODOLOGY

## 3.1 Generation of *SORL1-EGFP* reporter line

3.1.1 design guide RNA (gRNA)

The sequence of *SORL1* about 250 bp nearby stop codon was picked to design gRNAs by CRISPR design website (https://benchling.com/). The most two specific guides were chosen. Each gRNA was cloned into pX461 plasmid (a gift from Feng Zhang Lab; Addgene Plasmid #48140) and tested efficiency by T7 Endonuclease I

3.1.2 Donor vector construction

To generate donor vector, *SORL1* fragment was amplified by PCR and cloned into pGEM vector (pGEM®-T Easy Vector Systems - Promega Corporation) via TA cloning technique. The *EGFP* coding sequence with puromycin resistance gene was inserted into the vector as in-frame with *SORL1* which stop codon was extinguished by In-Fusion® HD Cloning Kit User Manual (Clontech). Afterthat the plasmid of *SORL1-EGFP* reporter was transformed into Stellar competent cells.

3.1.3 Transformation of *SORL1-EGFP* plasmid

The *SORL1-EGFP* plasmid 50 ng/ml were pipette gently into 50 µl of Stellar competent cells. A tube of mixture was placed on ice for 30 minutes, and then heated shock at 42 °C for 45 second before placing the tube on ice for 3 minutes. Then 1ml of SOC medium was pipetted into the tube of mixture before shaking at 200 rpm at 37 °C for 45-60 minutes. The cells were centrifuged at 5000 rpm for 5 minutes and the supernatant was removed. The pellet was spread on LB agar with final conc.100 µg/ml of ampicillin and incubated at 37 °C, overnight. The colonies were picked into LB broth and incubated at 37 °C, overnight. Plasmid were extracted via QIAprep® Miniprep before doing cut-check and sequencing. The accurate colony was cultured and extracted for large scale via QIAGEN® Plasmid purification kit.

3.1.4 Transfection of *SORL1-EGFP* into HEK 293 cells and clone selection

HEK293 cells were dissociated to single cell with 0.25% Trypsin-EDTA. Counting cells for $10^6$ cells mixed with targeting vector cocktail (gRNA-Cas9 vector and donor vector) in SF cell line buffer of 4D-Nucleofector® X. The mixture was transfected into cells though 4D-Nucleofector®. Transfected cells were cultured on DMEM Hight glucose with 10% FBS for 3 days. The puromycin was added into media at final concentration 1μg/ml for selecting cell colonies which have *EGFP* coding sequence in *SORL1* gene.

3.1.5 Transfection of *SORL1-EGFP* into iPSC lines and clone selection

Human iPSCs were dissociated into single cell by Accutase. The Single cells $10^6$ cells were mixed with targeting vector cocktail in P3 primary cell buffer of 4D-Nucleofector® X. The mixture was transfected into cells though 4D-Nucleofector®. Transfected cells were cultured on mTeSR1 medium for 3 days. The puromycin was added into media at final concentration 1μg/ml for selecting cell colonies which have *EGFP* coding sequence in *SORL1* gene.

**3.2 Human iPSC Generation**

3.2.1 A healthy donor and a patient who is in criteria and approved the consent form was collected blood for 10 ml (2 teaspoon) (IRB No. 107/62) by specialized medical or nursing staff that is holding medical license.

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3.2.2 Collect peripheral blood mononuclear cells (PBMCs) from whole blood

PBMC was collected by Ficoll-Paque™ Media - Density Gradient Media. Blood was diluted with 1XPBS (1:1). Ficoll was pipette into a new tube (ratio 1:3) and then gently overlaid Ficoll with diluted blood. The layer was centrifuged at 400g for 30 minutes without break. The solution separated for 4 levels; red blood cells, Ficoll solution, PBMC and PBS, respectively. The PBMC level was pipetted into a new tube

and washed with 1XPBS twice. The mixture was centrifuged at 1200 rpm for 5 minutes. The PBMCs were cultured in PBMC media.

3.2.3 Generation of iPSCs

The PBMCs were cultured in PBMC media for 6 days. Day 5, inactivated DR4 feeder cells were coated on disk 35 mm. Day6, the PBMCs about $10^6$ cells were transfected with plasmid mix 3 μg (pCXLE-hOCT3/4-shp53-F, pCXLE-hSK. pCXLE-hUL and pCXWB-EBNA1 (Addgene, Shinya Yamanaka Lab Plasmids) with P3 primary cell buffer of 4D-Nucleofector® X. Transfected cells were seeded on disk 35 mm coated inactivated DR4 on PBMC media. mTeSR1 medium was added on day 1, 3 and 5. Then, the media was changed to mTeSR1 until colonies were observed. Finally, the colonies of cells were picked.

## 3.3 Cell cultured

3.3.1 Human iPSC (hiPSCs)

The hiPSCs were cultured on Matrigel induced growth factors with mTeSR1 medium at 5% $CO_2$ at 37 °C in incubator. The cells were passaged with CTK solution for small clumps (10% collagenase type IV, 0.25% trypsin, 20% knockout serum, 1mM CaCl2 and ddH2O).

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3.3.2 Human embryonic kidney 293 cells: HEK 293 / 293FT cell line

HEK 293 /293FT were cultured in DMEM high glucose medium component with fetal bovine serum (FBS) 10%, L-Glutamine 1% and Antibiotic-Antimycotic 1% (gibco®, Thermo Fisher Scientific) at 5% $CO_2$ at 37°C in incubator. Cells were subcultured with trypsin-EDTA 0.25%. Media were changed every 2-3 days.

3.3.3 Human chronic myeloid leukemia (K562) cell line

K562 were cultured in RPMI 1640 Medium containing fetal bovine serum (FBS) 10%, L-Glutamine 1% and Antibiotic-Antimycotic 1% (gibco®, Thermo Fisher Scientific) at 5% $CO_2$ at 37°C in incubator. Culture media were changed every 2-3 days.

3.3.4 hiPSC-derived cortical neurons

3.3.4.1 Tetracycline-*Ngn2*-inducible

The *Ngn2* fragment was amplified by PCR and cloned into pDsRed-Express vector (Addgen plasmid 632412) to tagged *Ngn2* with RFP. Then amplified the fragment of *Ngn2*- DsRed-Express and ligated with linearized vector of Tet-on 3G inducible expression lentiviral system (Takara Bio USA, Inc). The large scale of Tetracycline-*Ngn2*-plasmid was extracted by QIAGEN® Plasmid purification kit.

3.3.4.2 Packing Lentivirus

293 FT cells were dissociated and seed into flask T75, 3 flasks before packing one day. The fresh media were change at least 3 hours before packing. Lentivirus were packing followed ProFection® Mammalian Transfection system protocol. The media were changed again in the next day. The media were harvested and precipitated at 25,000 rpm, 90 min at 4°C after 2 days later. Then removed the supernatant and resuspended with opti-MEM media 500 μl. Incubated the mixture at 4°C overnight and aliquot into microcentrifuge tube in the next day.

3.3.4.3 Infected tetracycline-*Ngn2*-inducible into *SORL1-EFGP* iPSCs

The iPSCs were dissociated into single cells and seeded into Matrigel coated dish before infection 1 day. Polybrene was added to fresh media to final concentration 1x before changing media to the cells. Lentiviral was dropped into the dish and mixed softly. The infected cells were incubated at 5% $CO_2$ at 37°C for 24 hours. Then cells were wash with 1xPBS at least 3 times before changing new fresh media.

3.3.4.4 iPSC-derived cortical neurons

The *SORL1-EFGP -Ngn2*-inducible iPSCs were treated with 2 μg/ml doxycycline for 48 hours. The treated doxycycline cells were dissociated into single cells and sorted PE (RFP) positive using untreated doxycycline for control. The sorted cells were washing-centrifuged at 80g for 10 minutes at least 4 times. The cells were seeded into 96 well plate 5,000 cells/well and 50,000 cells/well in 12 well plate coated with laminin. The RFP sorted cells were cultured with mTeSR1 media for the

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first day. The neurobasal media (DMEM/F12, neurobasal, human insulin, N2, B27, L-Glutamine, NEAA, 2-Mercaptoethanol and Sodium pyruvate) complement with BDNF, NT3, Y-27632 and laminin were changed in the next day with doxycycline for final concentration at 2 µg/ml. From day 6-9, the doxycycline was detached from media. The media were changed every 2 days.

**3.4 Cell Staining**

3.4.1 Immunofluorescence staining

Cells were fixed using 4% paraformaldehyde for 15 min at RT. The fixed cells were washed with 1xPBS twice, 5 minute each before blocking with 1xPBS buffer containing 0.3% Triton-X (Sigma-Aldrich) and 5% goat serum. Afterward, cells were incubated with primary antibodies overnight at 4 °C. Cultures were then washed with 1xPBS contain tween-20 for 3 times at least and incubated with secondary antibodies for 1 h at RT.  Primary antibodies used in this study were Nanog (1:100, D73G4, Cell Signaling), Oct-4A (1:100, C52G3, Cell Signaling), MAP2 (1: 5000, PCK-554P, BioLegend), Anti-**β**-Tubulin III (1:100, T8578, Sigma-Aldrich), Ctip2 (1:100, 25B6, abcam), SORL1 (1:100, D8D4G, Cell Signaling).

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3.4.2 Early endosome tracker

The reagent (CellLight™ Early Endosomes-GFP, BacMam 2.0, Invitrogen™) was directly added to the cells. The cells were incubated overnight. The transduced cells were observed in the next day.

3.4.3 Lysosome tracker

The media cells were removed from the well prewarmed (37°C) probe (LysoTracker™ Deep Red, Invitrogen™) containing medium was added. Incubate the cells for 30 minutes to 2 hours under growth conditions suitable for the cell type. Then replace the loading solution with fresh medium and observe the cells using a fluorescence microscope.

### 3.5 BDNF and cAMP treated

iPSCs-derived neurons were treated with BDNF (biotechne, 50 ng/ml) or cAMP (250 μg/ml) for 48 hours. Then cells were harvested for RNA using the RNeasy kit (QIAGEN).

### 3.6 CRISPR-on sgRNA generation

3.6.1 Designed single guide RNA

Single guide RNAs were designed from the 330 bp upstream of transcriptional start site (TSS) of *SORL1* sequencing following Zhang's protocol by specific website for CRISPR gRNA (https://benchling.com/) (table3). The gRNAs were cloned into pAC154-dual-dCas9VP160-sg Expression (a gift from Rudolf Jaenisch, Addgene plasmid # 48240).

3.6.2 sgRNA transfection

The sgRNASORL1 plasmid was transfected into cells at total concentration 3 μg/ml per cell $10^6$ cells at least. The fresh medium was changed in the next day. Transfected cells were harvested for 48 hours.

### 3.7 RNA extraction and quantitative real time-PCR (qRT-PCR)

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Cell cultures were treated with Trizol reagent for total RNA extraction. qRT-PCR was performed using SYBR, Thermo Fisher Scientific. The qRT-PCR data were analyzed using double delta Ct value (**ΔΔ**Ct). GAPDH was used as reference gene. The following primers were shown as table1

### 3.8 Statistic

All data were presented as mean ± SD. Normally distributed data were analyzed using two-tailed unpaired t tests. Statistical analyses were performed using Prism software (GraphPad). Group differences were considered statistically significant at *P<0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

*Table 1* List of Primers

| Primers | Forward | Reward |
|---|---|---|
| SORL1 | AAGTGGGTGTGTTACGAGGC | AGAACTCTCCCCCACAACCA |
| T7 for SORL1 | CTGGGGAACCCAAACTACCC | ACCTGGTTTGAAGTTCACAGT |
| pGEM-SORL1 linearized | AAGAGCTTTCCTCACTAGAAACCAAAT | GGCTATCACCATGGGGACGTCATCTGA |
| EGFP-PGK-Puro | CGTCCCCATGGTGATAGCCATGGTGAG CAAGGGCGAGGAGCT | TCTAGTGAGGAAAGCTCTTATAACTTCGT ATAGCATACATTAT |
| Ngn2-dsRed express | TCTATCGATGGATCCGCCACCATGTTCG TCAAATCCGAGACCTT | GAGGGGCACGGATCCCTAGATACAATCC CTGGCTATG |
| TRE3G-Ngn2 | GTCTTATACTTGGATCGCCACCATGTTC GTCAAATCCGAGACCTTG | CTACCCGGTAGAATTCCTACAGGAACAG GTGGTGGCGGCC |

*Table 2* List of Primers for qRT-PCR

| Primers | Forward | Reward |
|---|---|---|
| GAPDH | CTGGGCTACACTGAGCACC | AAGTGGTCGTTGAGGGCAATG |
| SORL1 [9] | CCAACCTGAAGATGGGTCATA | ACAGCAGCAACATCCGTAGAT |
| Ngn2 [33] | CGCATCAAGAAGACCCGTAG | GTGAGTGCCCAGATGTAGTTGTG |
| Tbr2 [34] | CACCGCCACCAAACTGAGAT | CGAACACATTGTAGTGGGCAG |
| Map2 [34] | CTCAGCACCGCTAACAGAGG | CATTGGCGCTTCGGACAAG |
| Ctip2 [34] | GAGTACTGCGGCAAGGTGTT | TAGTTGCACAGCTCGCACTT |

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*Table 3* List of CRISPR gRNA

| gRNA | sequences |
| --- | --- |
| SORL1 reporter guide1 | TCTTTCAGGCTATCACCATGGGG |
| SORL1 reporter guide2 | GCTCTTTCAGGCTATCACCATGG |
| sgRNASORL1-1 | TTCCTGCAGGGAGAACAAGG |
| sgRNASORL1-2 | CAGGAATGAAGAGTTGCATG |
| sgRNASORL1-3 | CCGAACCGAGCGGGACCTGG |
| sgRNASORL1-4 | TCTGCGCTCGGCGGCCTCGG |
| sgRNASORL1-5 | GGGGTGCGTCCCTTCCCTGG |
| sgRNASORL1-6 | GCTGCGAGCCTCACACGTGA |
| sgRNASORL1-7 | TCCGGCCCCTCCCTGGCGCG |
| sgRNASORL1-8 | AGCGCAGAAAGTGCGCGAAA |
| sgRNASORL1-9 | ACTTCCCCATCCCCGCGCCA |
| sgRNASORL1-10 | GCGCGGCGCCGTCACGTGTG |

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# CHAPTER IV

# RESULTS

## 4.1 Generation of *SORL1-EGFP* reporter line in HEK293 cells

To our knowledge *SORL1* is associated with directing APP to endocytic pathways, retromer-dependent retrograde trafficking and be involved in the process of sorting toxic Aβ oligomers for degradation at lysosome. Overexpression of *SORL1* has been shown to impair amyloidogenic process [9]. However, the regulation of *SORL1* in AD is still unknown. Thus, to generate *SORL1-EGFP* reporter to monitor the *SORL1* level in human neurons by using iPSCs together with genome editing, CRISPR-Cas9 technology. Two guide RNAs were designed to bind to the DNA in the location closest to the stop codon of the *SORL1* gene (Figure 1A). Both guides were tested for efficacy and the precision, combined with the Cas9 protein, induces DNA double strand breaks (DSBs) at the *SORL1* gene in HEK293 cells by the T7 endonuclease assay. The results demonstrated that T7 endonuclease was able to cut DNA at 375 bp and 211 bp in the first guide RNA group and 377 bp and 209 bp in the second guide RNA group, respectively. The DNA strand was not cleaved in the non-guide RNA group (Figure 1C), indicating that both guided RNAs were able to induce DNA strand break in the *SORL1* gene nearby the stop codon. In order to generate *SORL1-EGFP* reporter using guide RNA and Cas9 protein to induce DNA strand break in the desired location, donor vector is needed to act as the DNA template. The donor vector was generated by cloning the DNA of *SORL1* at the last exon (exon 48) with the destruction of the codon position, and then following the *EGFP* coding sequence, puromycin resistance gene was used to select cells for the *EGFP* coding sequence which was added to the *SORL1* gene (Figure 1B).

To test the efficacy of generated guide RNAs and donor vector, we directed targeting vector cocktails containing the guide RNA-protein Cas9 vector and *SORL1*-

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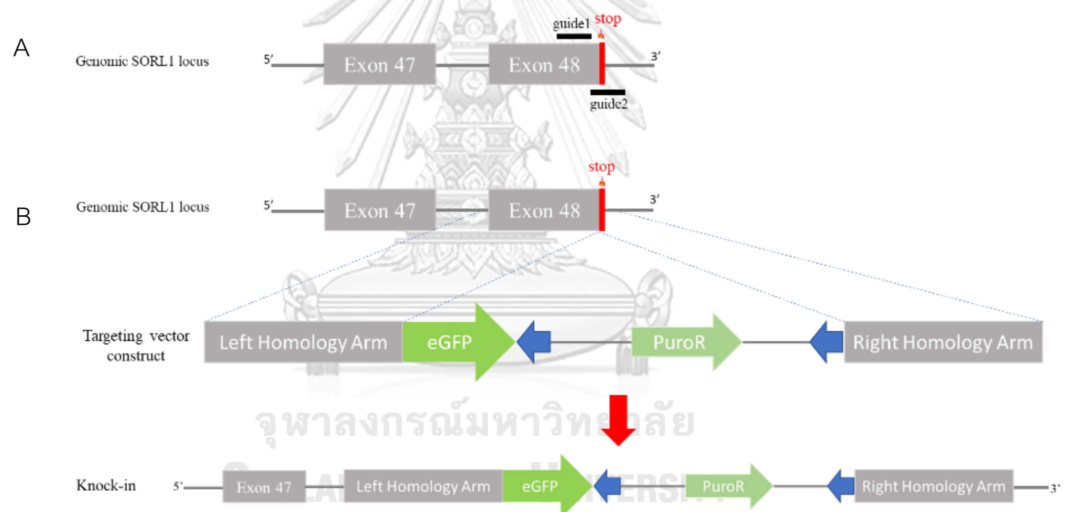
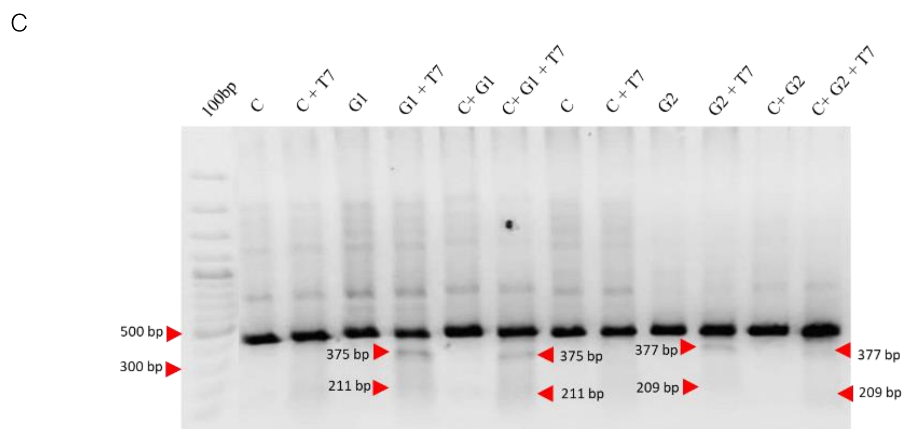
*EGFP* donor vector to HEK293 cells through the electroporation technique. The *EGFP* coding sequence infiltrated cells were selected using puromycin. After selecting puromycin-resistant HEK 293 cells, the insertion of *EGFP* coding sequence were examined by PCR technique. The results showed that the cooperation of gRNAs, Cas9 protein and donor vector was able to induce *EGFP* fusion with endogenous *SORL1* (Figure. 1D). Observing *EGFP* expression in selective cells using live cell imaging technique, *EGFP* expression was found in the cytoplasm of the cell (Figure 1E). The position of *EGFP* expression was the same as that of *SORL1* expression in the preceding in HEK293 cells and the accuracy is sufficient to be verified in iPSCs which is more difficult to edit genes.



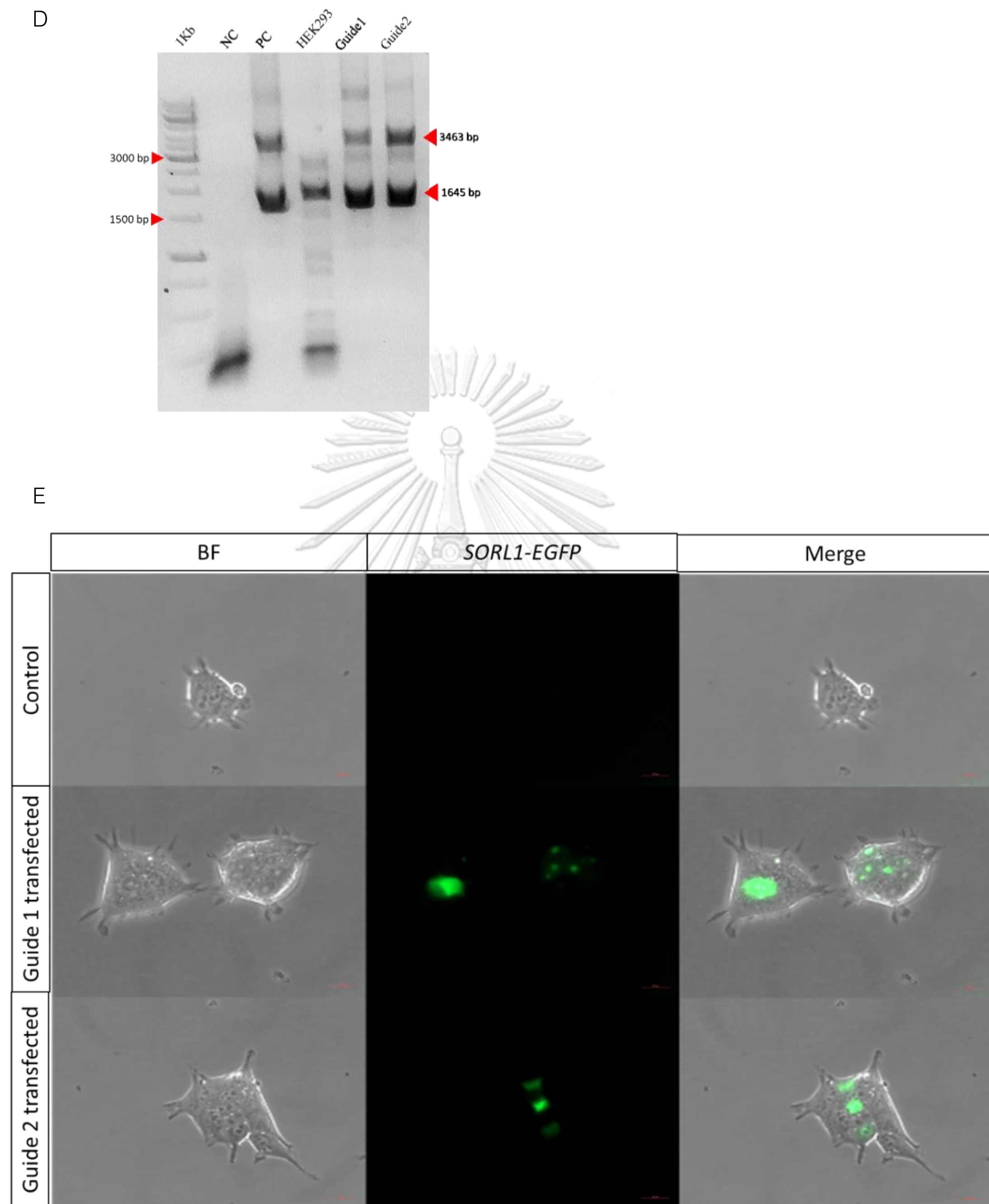



**Figure 1** Diagram of gRNAs and donor vector.

(A) represented the cutting location on *SORL1* gene (B) diagram of *SORL1-EGFP* donor vector. (C) gRNAs efficacy tested via T7 Endonuclease I induce nucleotide clavation (C = control, G1 = Guide1, G2 = Guide2 and T7 = T7 Endonuclease I) (D) confirming the *EGFP* coding sequence insertion by

PCR (NC = negative control and PC = positive control). (E) Live cells represented *EGFP* in HEK293 cells under fluorescence microscope.

### 4.2 *SORL1-EGFP* reporter iPSC lines were successfully generated

To generate iPSC line, a control iPSC line from healthy was generated (Figure 2A). Characterization of iPSCs were declared to success reprogramming of PBMC using immunofluorescence staining. The pluripotent markers as *Nanog* and *Oct4* were expressed (Figure 2B). The generation of the iPSC line from the healthy was successful.

To generate the *SORL1-EGFP* reporter iPSC line, the targeting vector cocktails were electroporated into control iPSCs. After screening of cell colonies using puromycin, *EGFP* coding sequence was confirmed by PCR and the nucleotide sequence was validated by DNA sequencing technique, it was found that the *EGFP* coding sequence was inserted into the appropriate location of the *SORL1* gene in control-iPSC (Figure 2C). It did not cause any alteration in the expression of *Nanog* and *Oct4* genes, which were related to the pluripotent property determined by immunofluorescence staining (Figure 2D). Moreover, the *SORL1-EGFP* could express in iPSC phase under live cell imaging (Figure 2E).

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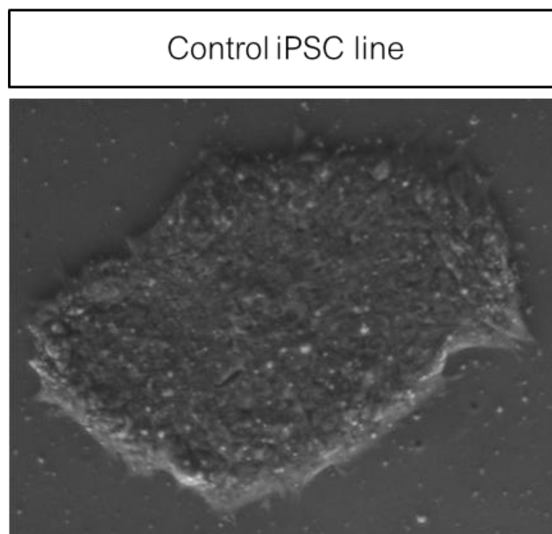

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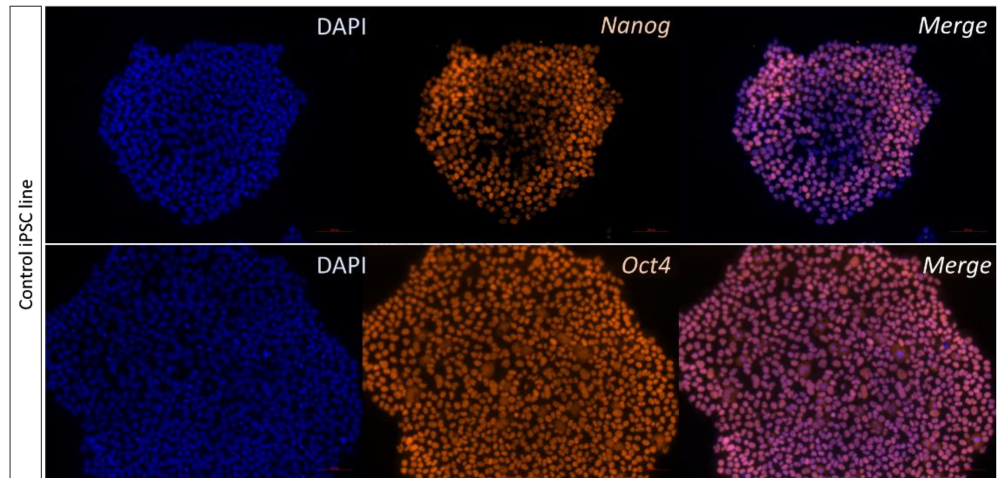


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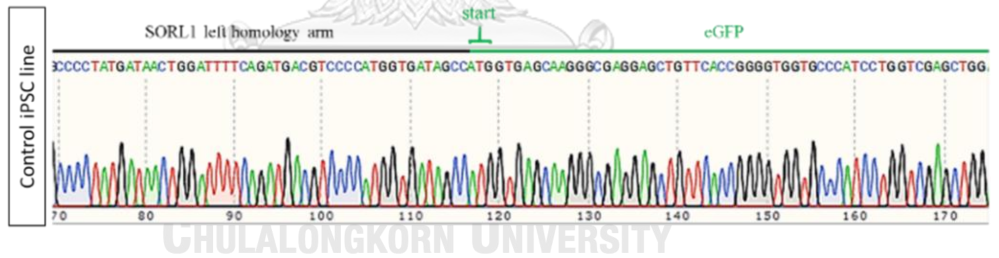


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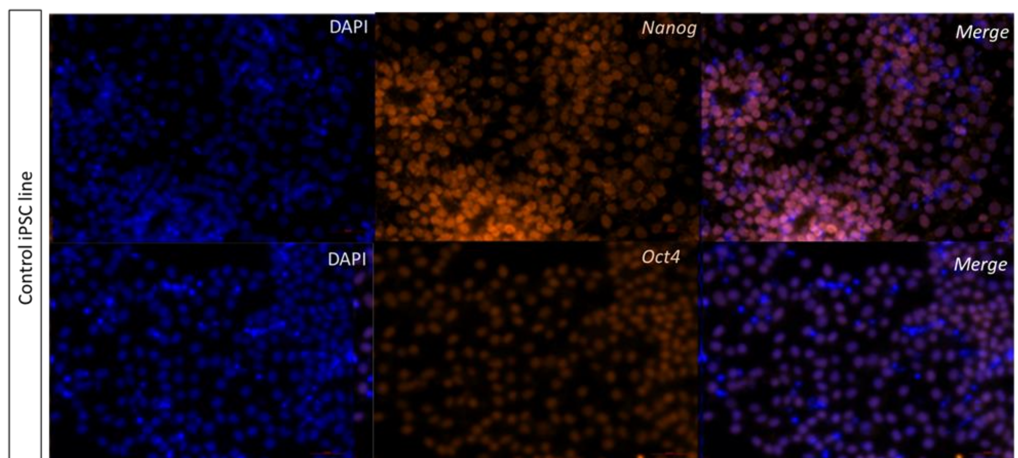

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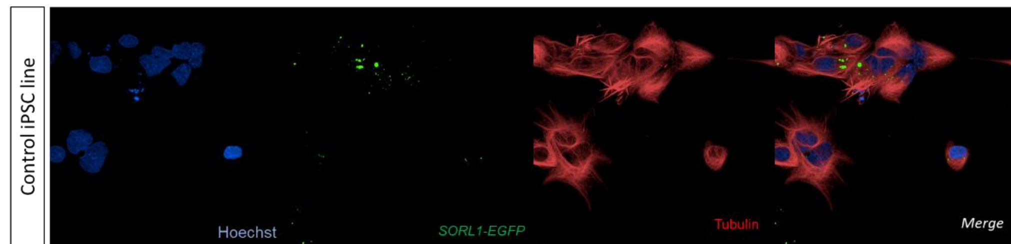


**Figure 2** Generation of iPSCs line and SORL1-EGFP reporter line.

(A) Colony morphologie of control iPSC line (bright field) (Scale = 200 μm). (B) Immunofluorescence staining of pluripotent stem cell markers, *Nanog* and *Oct4* in control iPSC line (Scale = 200 μm). (C) The DNA sequencing analysis was confirmed the *EGFP* nucleotide which inserted in *SROL1* gene in control-*SORL1-EGFP* reporter iPSC line. (D) The cells were maintaining pluripotency properties in *SORL1-EGFP* reporter iPSC line as express *Nanog* and *Oct4* using immunofluorescence staining (Scale = 200 μm). (E) The *SORL1-EGFP* could express in control iPSC line in iPSC phase.

### 4.3 Optimizing neuronal differentiation protocol

We next examined whether control-*SORL1-EGFP* line could develop into neural lineage by following direct conversion technology to differentiate human iPSCs into cortical neurons using lentiviral induction of neurogenin2 (*Ngn2*) [10, 35, 36]. Here, tetracycline inducible *Ngn2* was created and used (Figure 3A). The *Ngn2* gene was activated to overexpression after adding doxycycline. The results demonstrated that *Ngn2* gene were slightly express RFP positive cells after turning doxycycline for 24h under fluorescence microscope (Figure 3B). This result corresponds to the RFP detection on flow cytometer. It was displayed that *Ngn2* expressed 64.4% in control iPS cell lines (Figure 3C). After 8 days differentiated in cortical differentiation media. The cortical neuron cells were low purity and iPSC colonies were found in this condition (Figure 3D). To purify, the differentiation protocol was adapted. The *Ngn2*-

RFP positive cells were sorted after turning on doxycycline 2 days. The sorted cells were recovered in mTeSR1 for 1 day before differentiated into neurons directly. The morphologies of cells were changed to neural in day2 and the cells could proliferate observed by increasing the number of cells in the cell culture containers in day5 of differentiation (figure 3E). The differentiated cells were high purity (≥90%) under this protocol. Thus, control *SORL1-EFGP-Ngn2*-inducible iPS cell line could develop to neural lineage after 9 days cultured in the present of doxycycline.

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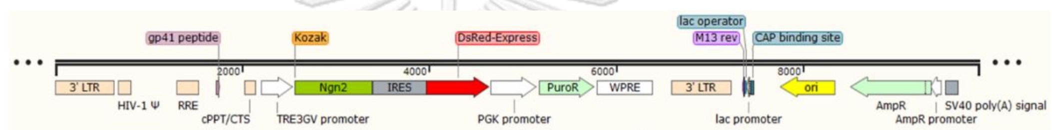


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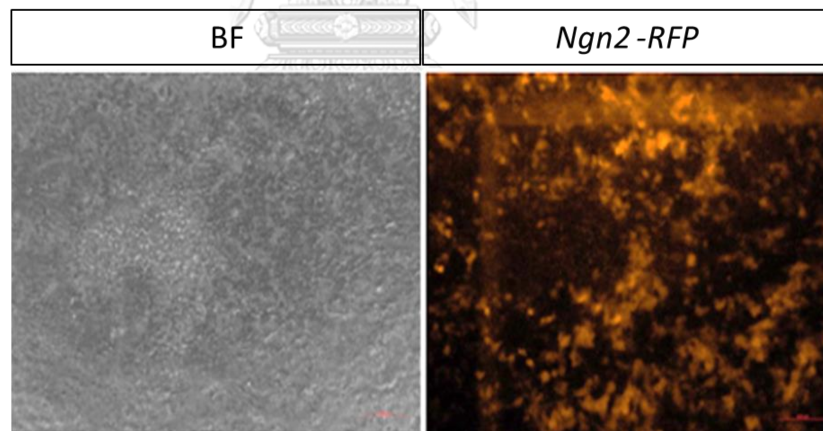

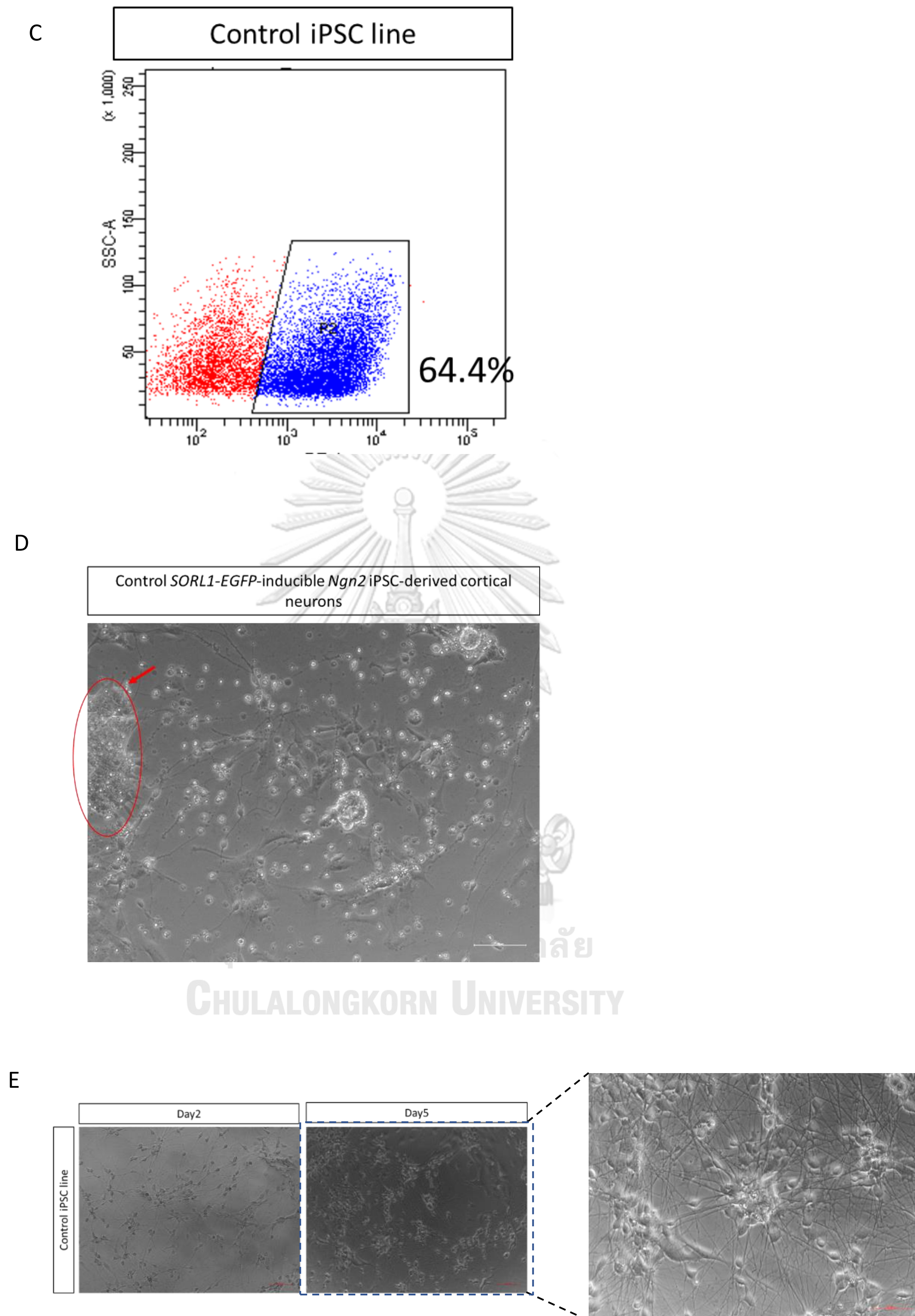


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**Figure 3** The tetracycline inducible Ngn2 system and *SORL1-EFGP*-inducible *Ngn2* iPSCs-derived neural cells

(A) Component of tetracycline inducible Ngn2. (B) Ngn2-RFP positive cells were observed under fluorescence microscope after turning doxycycline for 24h. (C) Flow cytometry analyzed of Ngn2-RFP positive in control iPSC line. (D) The differentiated cells were low purity of neuron cells and iPSC colonies were observed. (E) Morphological of control iPSCs-derived cortical neurons in day2 and 5 of differentiation (Scale = 100 µm).

### 4.4 Characterization of *SORL1-EFGP*-inducible *Ngn2* iPSC-derived cortical neurons and *SORL1-EGFP* expression pattern

To characterize the *SORL1-EFGP-Ngn2*-inducible iPSC-derived cortical neurons. The cortical neurons were stained with neural marker (MAP2 and Ctip2) at day 9. The results indicated that *SORL1-EFGP*-inducible *Ngn2* iPSC-derived cortical neurons were expressed MAP2 and Ctip2 as shown as Figure 4A. In order to determine the expression profile of the differentiated cortical neurons, those cells were expressed MAP2 (neuron marker) and TBR1(cortical neurons) (Figure 4B). Moreover, *SORL1-EGFP* expression pattern were presented as dots in cytoplasm. The *SORL1-EGFP* expression pattern were similar to SORL1 immunofluorescence stanning (Figure 4C).

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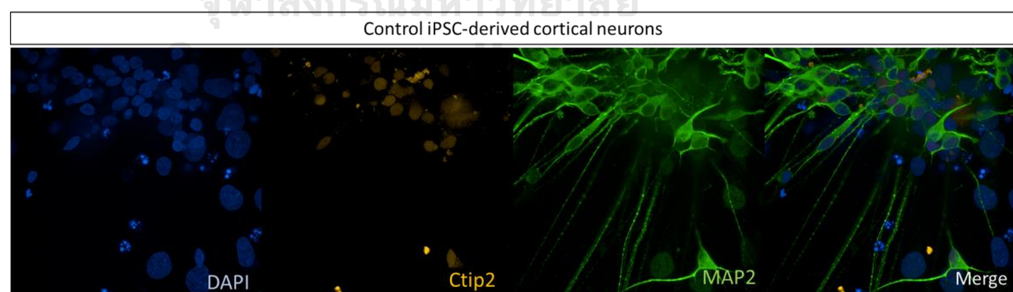

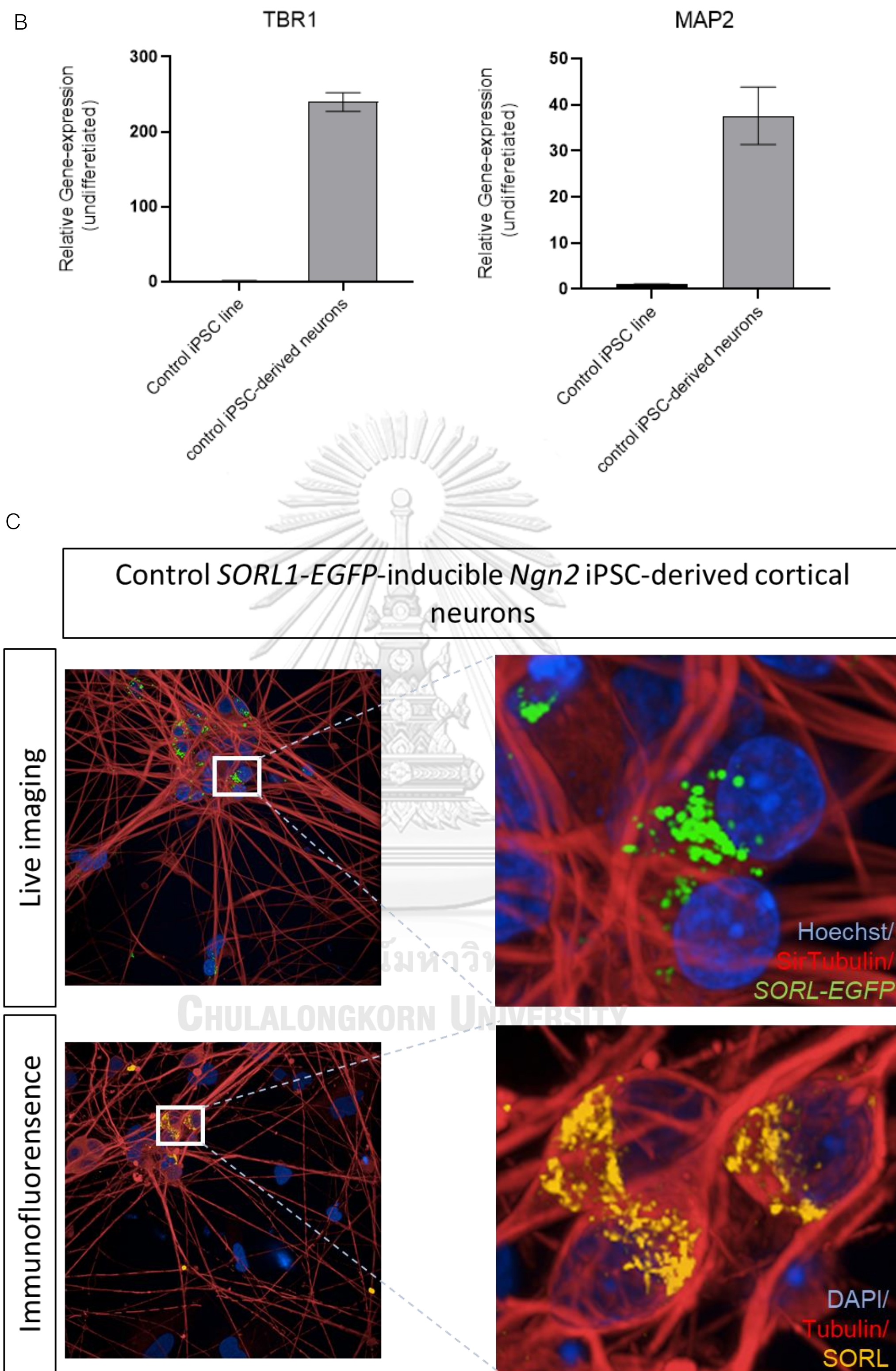


**Figure 4** Characterize the *SORL1-EFGP* -inducible Ngn2 iPSC-derived cortical neurons.

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(A) The cortical neurons were expressed neural marker (Beta III tubulin and MAP2). (B) qRT-PCR analyzed that cells were expressed neural marker (MAP2) and cortical neuron (TBR1). (C) SORL1-EGFP expression pattern were similar to SORL1 immunostaining.

### 4.5 The *SORL1-EGFP* in the control iPSC-derived neurons were localized correctly

According to understand that *SORL1* is associated with directing APP to endocytic pathways and be involved in the process of sorting toxic Aβ oligomers for degradation at lysosome. To ensure the location of *SORL1-EGFP* in the control iPSC-derived neurons, the *SORL1-EFGP* -inducible *Ngn2* iPSC-derived cortical neurons were stained with bacurovirus targeting at RAB5a where is specific to early endosome (early endosome marker) and lysotracker (lysosome marker). We observed that *SORL1-EGFP* co-localized with early endosome and lysosome (Figure 5A) under live cell imaging. Interestingly, to test activation of *SORL1-EGFP*, the *SORL1-EFGP* -inducible *Ngn2* iPSC-derived cortical neurons were treated with BDNF and cAMP which induce *SORL1* expression. The results established that the *SORL1-EGFP* were more expressed in the BDNF and cAMP condition compared to untreated condition observing under florescence microscope (Figure 5B). The *SORL1-EGFP* expression were correlated with the qRT-PCR analysis. The graph of gene expression was presented that *SORL1* expression levels were significantly increased in BDNF and cAMP treated to 0.3-fold and 0.5-fold, respectively (Figure 5C). Suggesting, the iPSC *SORL1-EGFP* reporter lines were generated effectively. The *SORL1-EGFP* could show the location and activities of *SORL1* in cells.

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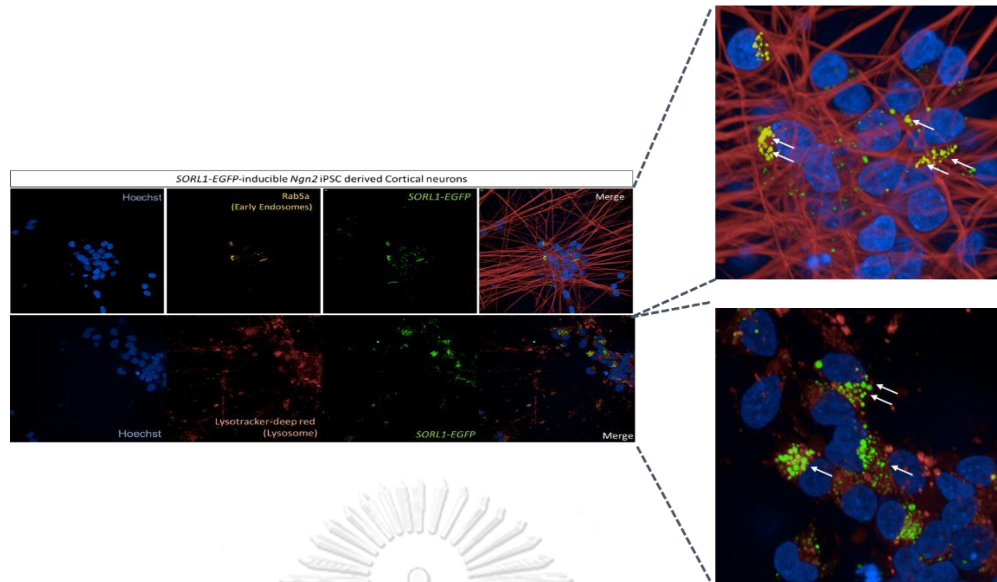


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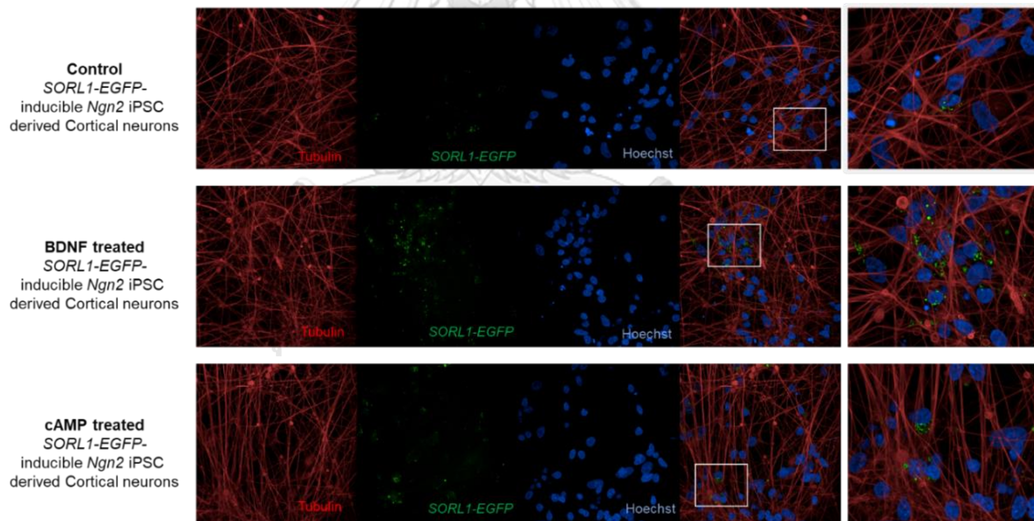

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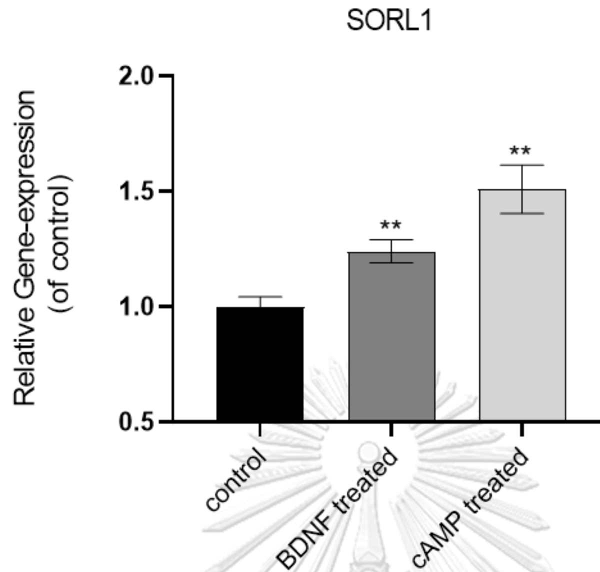


**Figure 5** Ensuring the location of *SORL1-EGFP* in the cells and activation of *SORL1-EGFP*
(A) Live cell imaging showed that the *SORL1* co-localized with early endosome and lysosome under live cell imaging. (B) The *SORL1* were more expressed in the BDNF and cAMP condition observing under florescence microscope. (C) SORL1 expression level after treating BDNF and cAMP by qRT-PCR. All values represent mean ± SD. Normally distributed data were analyzed using two-tailed unpaired t tests. (**P < 0.01).

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## 4.6 High-Throughput drug screening using *SORL1-EGFP* iPSC derived cortical neurons

To test *SORL1-EGFP* iPSC-derived cortical neurons with High-throughput drug screening system. High throughput confocal imaging with imaging analysis were used to measure size, intensity and number of spots of *SORL1-EGFP* per cell. The *SORL1-EGFP* iPSC derived cortical neurons were treated with Aβ42. We observed enlarged spot of *SORL-EGFP* and the spot expression were increased (Figure 6A). It might according to SORL1 activity in endocytic and degradation pathway when the Aβ42 level increased.

In addition, *SORL1-EGFP* reporter line was generated in SAD-iPSC line. The SAD-iPSC was reprogrammed from PBMC of a sporadic AD patient with a novel *SORL1* mutation (c.2603C>A) with heterozygous *APOE* (c.388T>C). The results established that the *SORL1-EGFP* SAD-iPSC cortical neuron were generated successfully. The *SORL1-EGFP* expression pattern in this line was similar to *SORL1-EGFP* control-iPSC cortical neuron (Figure 6B). The *SORL1*-mRNA expression level were not different from both control iPSC line and *SORL1-EGFP* control-iPSC cortical neuron (Figure 6C). Conversely, the High throughput confocal imaging with imaging analysis illustrated that size of spot of *SORL1-EGFP* in *SORL1-EGFP* SAD-iPSC cortical neuron were larger than size of spot of *SORL1-EGFP* in *SORL1-EGFP* control (WT)-iPSC cortical neuron (Figure 6D). The effect of the *SORL1* novel mutation is required to more clarify.

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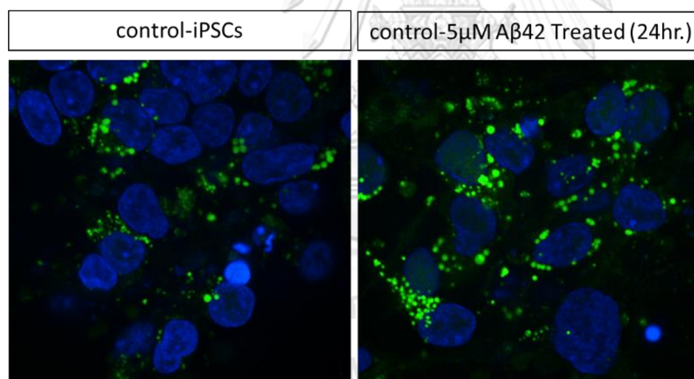


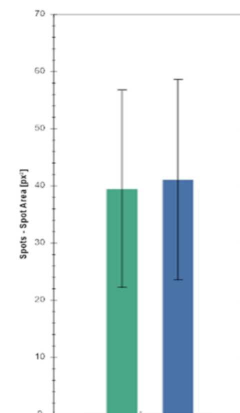


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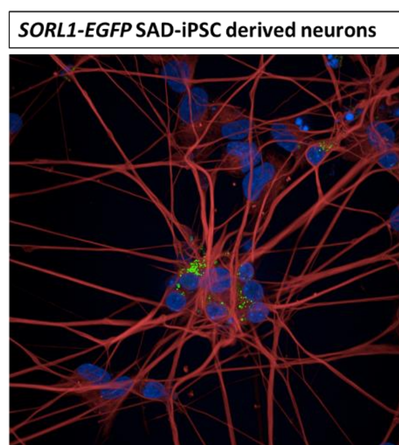

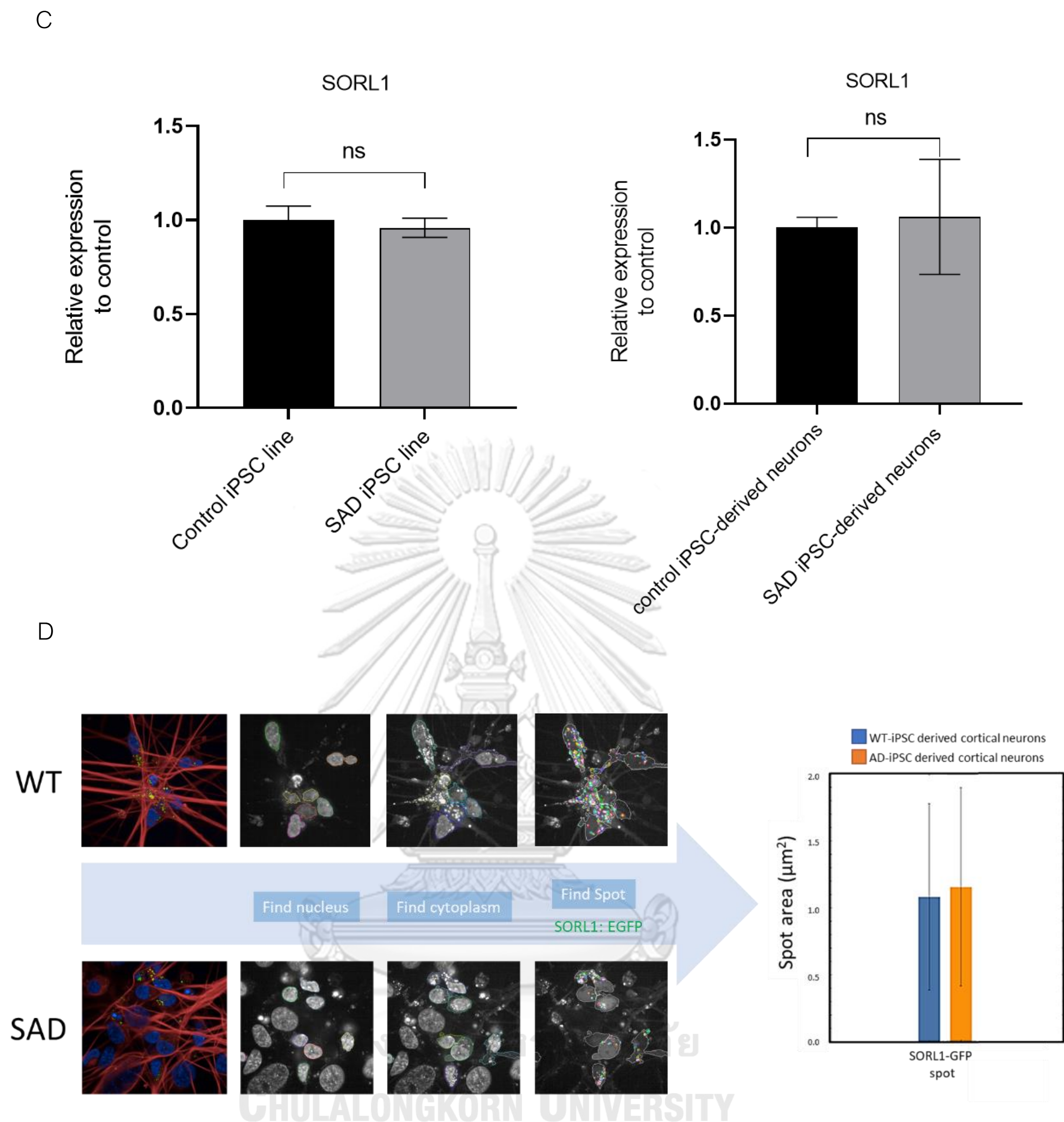


**Figure 6** High-Throughput drug screening using SORL1-EGFP iPSC derived cortical neurons (A) Enlarged spot of SORL-EGFP in WT treated with 5μM of Aβ42. (B) The SORL1-EGFP expression pattern in this line was similar to *SORL1-EGFP* control-iPSC cortical neuron. (C) The *SORL1*-mRNA expression level were not different from both control iPSC line (ns= 0.4471) and *SORL1-EGFP* control-iPSC cortical neuron (ns= 0.7702). All values represent mean ± SD. Normally distributed data were analyzed using two-tailed unpaired t tests. (D) The size of spot of *SORL1-EGFP* in *SORL1-EGFP* SAD-iPSC cortical neuron were larger than size of spot of *SORL1-EGFP* in *SORL1-EGFP* control (WT)-iPSC cortical neuron.

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**4.7 Whether CRISPR-on could increase the *SORL1* expression in cell lines**

The growth factor or small molecule as BDNF or cAMP could induce *SORL1* expression, those may perhaps slightly activate the *SORL1* expression. Whether overexpression could increase the *SORL1* level. Although, overexpression is more effective to stimulus, it is more difficult especially for gene therapy because the size of cassette and it random integrated [37]. The CRISPR-on technique was reported to activate high specific endogenous and it was successful to drive gene expression *in vitro* [37-39] . To unravel, CRISPR-on system might be a good choice. Ten sgRNAs were designed to target the endogenous human *SORL1* gene (Figure 7A) and confirmed the sequence by sequencing after cloning.

Afterwards ten CRISPR-on were validated sgRNA binding to upstream region of the transcriptional start site (TSS) of *SORL1* and tested their transactivation activity in K562 cells which is low or do not express *SORL1* in cells. To identify the most efficient of binding site, each sgRNA was transfected into K562 cells. The results indicated that sgRNASORL1-3 is the strongest activation which increased *SORL1* expression up to 400-fold, followed by sgRNASORL1-4, sgRNASORL1-1 as 40-fold and 10-fold, respectively. *SORL1* expression slightly change in sgRNASORL1-2 and sgRNASORL1-10 as 7-fold and sgRNASORL1-5,7-9 as 3-4-fold. The lowest activation expression is sgRNASORL1-6 which the level of *SORL1* expression was equivalent with control showed in Figure 7B. This data suggested that sgRNASORL1-3 was the most proficiency activation transcriptional of *SORL1*.

A combination of three sgRNAs was suggested that was the most efficient to activate the targeting genes [40]. According to this understanding, the combination of three sgRNASORL1s were set to 6 groups as group1 is the best three sgRNASORL1s, group2 was the worse three sgRNASORL1s, group3 was the nearest three sgRNASORL1s to TSS, group4 was the farthest three sgRNASORL1s to TSS, group5 was the best three sgRNASORL1s which are on the forward stand and the last group was the best three sgRNASORL1s which were on minus stand. An *SORL1* expression over 100-fold had 3

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groups as 600-fold, 350-fold and 200-fold activation were achieved with sgSORL1-1,3,4 (group1), sgSORL1-1,3,8 (group5) and sgSORL1-3,6,10 (group4), respectively. While *SORL1* expression in other 3 groups are less than 100-fold as an 80-fold, 40-fold and 14-fold activation with sgSORL1-2,4,10 (group6), sgSORL1-1,2,5 (group3) and sgSORL1-5,6,7 (group4), respectively (Figure 7C). These data suggest that combination of three guides were more efficient than one guide.

To test double activation of CRISPR-on, the combination group 3 was regrouped into double combination to 3 groups as sgRNASORL1-1,3 in group 1, sgRNASORL1-1,4 in group2 and sgRNASORL1-3,4 in group3. As presented in Figure 7D, *SORL1* was upregulated to 500-fold in group1, 70-fold in group2 and 300-fold in group3. Thus, in this study double combination of sgRNASORL1-1,3 was probably high efficiency to activate SORL1 gene in K562 cell line. According to above tissue, sgRNASORL1 might be successful to motivate *SORL1* in iPSC-derived cortical neurons for a disease model and this tool might be applied to cure the AD patient in the future.

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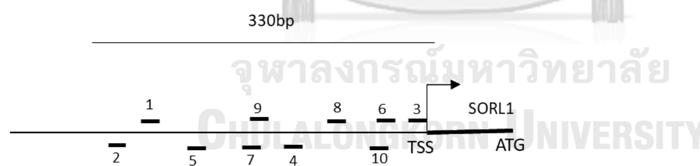


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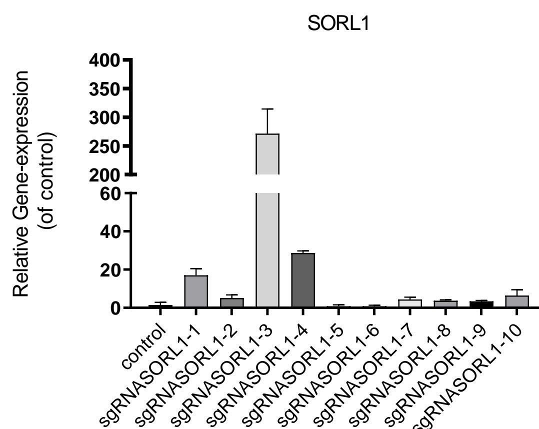


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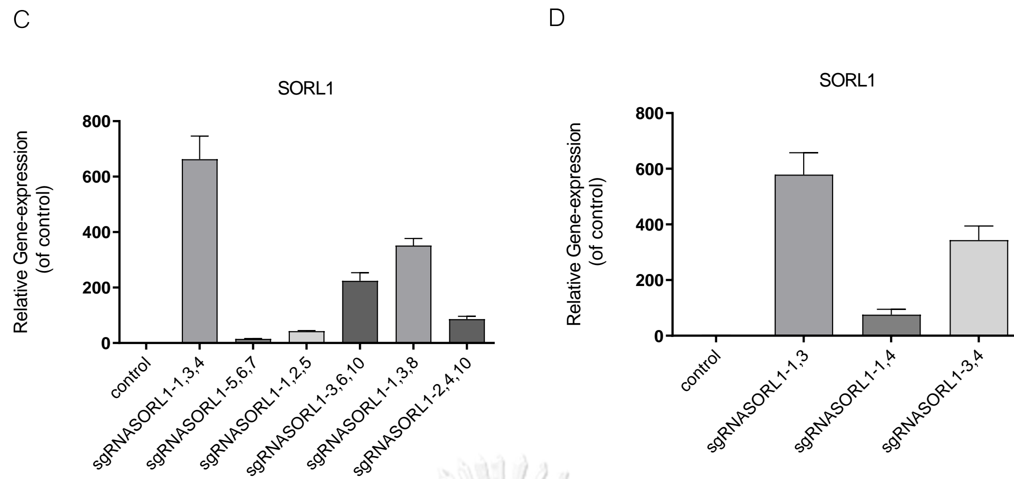


Figure 7 The location of sgRNAs on the 330bp upstream of transcription start site of SORL1 and validation of single, double and triple sgRNAs for dCas9VP160-mediated transcriptional activation.

(A) The location of sgRNAs on the 330bp upstream of transcription start site of *SORL1*. (B) The quantitative measured the *SORL1* expression levels in validation single sgRNA, (C) Triple combination of sgRNA and (D) Double combination of sgRNA, respectively

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# CHAPTER V

# DISCUSSION

Neurodegenerative disease modeling using iPSCs has provided novel opportunities for elucidating disease mechanisms and identifying potential therapeutic intervention[41-44]. Endosomal trafficking defect has recently been proposed to play important roles in early Alzheimer's disease pathogenesis[45]. SORL1 is a risk gene of Alzheimer's disease which regulate neuronal endocytic sorting and APP processing [46]. In this study, we engineered *SORL1-EGFP* knock-in iPSC lines with doxycycline-inducible *Ngn2* expression cassettes [47, 48]. We established that engineered iPSC lines, after differentiated cortical neurons were induced by doxycycline, rapidly produce neurons with *SORL1-EGFP* positive dots that strongly colocalized with both early endosome and lysosome markers in the soma consistent with known *SORL1* role in endosome sorting. Importantly, *EGFP* signals increased upon treated with known upstream regulator of *SORL*1 such as cAMP and BDNF [9]. It leads to find a tool for screening compounds that enhance SORL1 expression in the endosome-mediated degradation pathway. Since mounting evidence suggest that endosomal transport defects could also be a key pathologic step in other neurodegenerative diseases such as ALS, Parkinson's disease, and Huntington's disease [49, 50], this platform could have even broader applications.

We generated *SORL1-EGFP* iPSC reporter cell line form sporadic AD patient with novel *SORL1* mutation. Preliminary result indicated that neurons produced from this iPSC line increased amyloid beta 42 secretion compared to control iPSC line. This finding is similar to previous reports which show *SORL1* disruption [51] or reduced expression [9, 52] in iPSC derived neurons resulted in an increase in amyloid peptide in culture supernatant and suggests that our novel *SORL1* mutation is loss of function. Nevertheless, due to heterogeneity between different iPSC lines, more tests are

needed to be performed to confirm this conclusion. To improve upon our report system, we are in the process of using CRISPR to edit our iPSC reporter lines to generate isogenic lines carrying different *SORL1* promoter mutation. Previous report has demonstrated that different mutations in *SORL1* promoter can distinctively affect level of *SORL1* expression and its responsiveness to specific stimuli, therefore may contribute to differential risk and drug responsiveness among patients with *SORL1* mutation [9]. We are also interested in using CRISPR to create isogenic *SORL1-EGFP* reporter lines with mutations responsible for familial AD such as APP, presenilin 1 [53] and sporadic AD risk gene *APOE4* [54, 55]. Moreover, we are working with our collaborator to incorporate new APP cleavage tracing technique into our platform [56]. Thus, we believe that this tool would become useful for AD research communities in the near future.

Over the last decade gene therapy has progressed remarkably and started to yield results in various animal model of CNS disorders. Although *SORL1* overexpression confer protective effect against amyloid toxicity, direct gene therapy approach with full *SORL1* expression cassette is technically challenging due to the large size of *SORL1* ORF. In this study, we succeed in creating combination of gRNAs that can effectively turn on endogenous *SORL1* in cell line. We are now in the process of creating AAV virus containing these gRNAs and CRISPR-VP160 cassette to test our strategy in *SORL1-EGFP* iPSC-derived cortical neuron to test its effectiveness. Nevertheless, with many efficient CRISPR delivery systems for CNS disorders show promising result in animal models [57-60], our guide RNAs in CRISPR-ON system has potential to develop into viable therapeutic strategy for AD.

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