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이학석사학위논문

Gamma-secretase 새로운 활성조절 유전자의 동정 및 기능 규명 연구

Isolation and characterization of novel gamma-secretase regulator

2017년 8월

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지도교수 정 용 근

이 논문을 이학석사 학위논문으로 제출함
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2017년 6월

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부 위 원 장 정 용 근 (인)
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ABSTRACT

Isolation and characterization of novel gamma-secretase regulator

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Alzheimer's disease (AD) is primarily common neurodegenerative disorder represented by cognitive deficit and memory loss. Amyloid plaques and the neurofibrillary tangles, which are generated by the accumulation of amyloid beta (Aβ) peptide outside neuron and hyperphosphorylated tau protein inside neurons respectively, represent pathological hallmark of AD. Sequential cleavage of APP (amyloid precursor protein) by beta (β)-/gamma (γ)-secretase is required for Aβ production. The γ-secretase, high molecular weight enzyme complex, includes four core proteins and its activity is the rate-limiting step of the reaction. Although γ-secretase has important role in Aβ generation, the regulatory action on γ-secretase complex for Aβ generation has not much been resolved at the

molecular level. Thus, it is important to better understand molecular regulation of γ-secretase and isolate new modulator of γ-secretase that is critical for the understanding and treatment of Alzheimer's disease. Here, by using genome-wide and cell-based functional screening system, I isolated IRF2 as a novel modulator of γ-secretase that stimulates Aβ production.

Ectopic expression of Interferon regulatory factor 2 (IRF2) enhanced γ-secretase-mediated cleavage of APP-CTFβ in *in vitro* AICD assay and increased Aβ production, leading to increase in aggregate prone $A\beta_{1-42}$ and thus change in the ratio of $A\beta_{1-40}/A\beta_{1-42}$. IRF2 expression facilitated the formation of an active γ-secretase complex by increasing nicastrin (NCT), anterior pharynx-defective phenotype 1 (APH-1) and active Presenilin 1 (PS1), the core components of γ-secretase. Together with overexpression analysis, knockdown of IRF2 expression reduced the formation of an active γ-secretase complex and γ-secretase activity. Interestingly, ectopic expression of IRF2 decreased Notch cleavage. Finally, other interferon regulatory family (IRF) also up-regulated γ-secretase activity. These results suggest that IRF2 is a selective activator of γ-secretase to increase Aβ generation by up-regulating the levels of holo γ-secretase mature protein complex.

Keywords: Alzheimer's disease, amyloid-beta (Aβ), gamma-secretase, IRF2

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ABBREVIATIONS

AD	**Alzheimer's disease**
Aβ	**Amyloid-beta**
AICD	**APP intracellular domain**
APP	**Amyloid precursor protein**
APH-1	**Anterior pharynx-defective phenotype 1**
BACE1	**β-site amyloid precursor protein cleaving enzyme**
BN-PAGE	**Blue-native PAGE**
DMEM	**Dulbecco's Modified Eagle Medium**
FBS	**Fetal Bovine Serum**
GFP	**Green fluorescent protein**
GOFS	**Gain-of-function screen**
HA	**Hemagglutinin**
IRF2	**Interferon-regulatory factor 2**
kDa	**Kilodalton**
LOFs	**Loss-of-function screen**
NCT	**Nicastrin**
NICD	**Notch intracellular domain**

PBS	**Phosphate buffered saline**
PEN2	**Presenilin enhancer 2**
PS	**Presenilin**
PMSF	**Phenylmethylsulfonyl fluoride**
mRFP	**Monomeric red fluorescent protein**
SDS-PAGE	**Sodium dodecyl sulfate polyacrylamide gel electrophoresis**
shRNA	**Small-hairpin RNA**

INTRODUCTION

Alzheimer's disease (AD) is progressive and fatal neurodegenerative disorder. Anatomically brain cortex shrivels up and these damaged areas are involved in thinking, planning, and remembering. Shrinkage is especially severe in the hippocampus, an area of the cortex that plays a key role in formation of new memories (Querfurth et al., 2010). The main symptoms of the disease are memory loss, cognitive impairment, and behavioral abnormalities caused by synaptic dysfunction and neuronal loss (Greene et al, 1996). Amyloid plaques generated from amyloid-beta (Aβ) fibrils presented outside neurons (Glenner et al., 1984; Masters et al., 1985), and neurofibrillary tangle made up of hyperphosphorylated Tau protein aggregates inside neurons (Hardy et al., 1998) are major pathological hallmarks of AD patient brain. Several lengths of Aβ peptides exist with the predominant form being $A\beta_{1\text{-}40}$. However, a longer form of Aβ, $A\beta_{1\text{-}42}$, is closely related with major toxicity at early stage of disease progression (Takami et al., 2009). In general, Aβ species cause major toxicity to neurons in the early stage of disease progression affecting Tau-mediated memory loss (Hardy et al, 2002). Thus, elucidation of the molecular regulation of Aβ production is critical for understanding of the pathogenesis of AD and slowing disease progression.

The Aβ is generated from sequential cleavage of Aβ precursor protein (APP) by beta (β)-site amyloid precursor protein cleaving enzyme (BACE1) and γ-secretase (Strooper et al., 1999; Roberson et al 2006). The γ-secretase is composed of at least four core subunits: Presenilin 1 or 2 (PS1 or PS2), nicastrin (NCT), presenilin enhancer 2 (PEN2) and anterior pharynx-defective phenotype 1 (APH-1) (Roberson et al., 2006; Selkoe et al., 2007; Periz et al., 2004). PS functions as a catalytic subunit of the enzyme complex and NCT is a gatekeeper of many substrates including APP, Notch, E-cadherin and ErbB4. APH-1 provides stability for assembly of the enzyme complex acting as a scaffold, forming a subcomplex with NCT, and PEN2 is linked to Presenilin endoproteolysis that is essential for activity of γ-secretase. The proteolytic activity of γ-secretase targeted to type 1 transmembrane proteins. Because γ-secretase processing is the rate-limiting step of Aβ generation, there have been many efforts to regulate activity of this enzyme complex in AD.

Until now, fewer than 10 regulators of γ-secretase have been identified, such as CD147, GPR3, and β-arrestin1 (Zhou et al., 2005; Ni et al., 2006; Liu et al., 2012). Of these, only GPR3 and β-arrestin1 selectively regulate γ-secretase substrate processing by exhibiting Notch-sparing activity. Furthermore, a small molecule has been identified that bind to APP to regulate APP processing and consequently increase the production of non-toxic forms of Aβ without causing

the side effects related with Notch deficiency (Kukar et al., 2008). These results raise the possibility that γ-secretase components, and/or substrate-targeting γ-secretase modulators are involved. Because the function of Notch is critical during animal development, the isolation of novel modulator of γ-secretase activity towards APP processing conserving Notch-sparing activity is required, which could be a attractive therapeutic target for drug development. Thus, despite massive efforts to understand Aβ production, discovery and identification of novel γ-secretase is important not only to advance the understanding of the gamma γ-secretase but also to find potential therapeutic targets.

The main purpose of this study is to isolate and characterize novel γ-secretase regulator candidates by using cell-based functional screening and to characterize the mechanism by which it can regulates γ-secretase.

MATERIALS AND METHODS

Antibodies

The following antibodies were used: anti-Actin (Sigma-Aldrich, A1978), anti-FLAG (Sigma-Aldrich, S7425), anti-GFP(Santa Cruz Biotechnology, sc-8334), anti-PS1-NTF (Santa Cruz Biotechnology, sc-7860), anti-APP-CTF (Sigma-Aldrich,A8717), anti-NCT (Sigma-Aldrich, N1660), anti-NICD (Novus, NB200-251), anti-CANX (Santa Cruz Biotechnology, sc-11397), anti-APH-1A and anti-PEN2 (kindly gifted from Dr. A. Takashima , RIKEN BSI, Japan)

Plasmid constructions

Human IRF2 was subcloned into p3x-flag CMV14. For IRF2 shRNA, target sequences are previously described in ref. (Ren et al., 2015; Yokota et al., 2017). The sequences for the construction of pSuper-puro-shIRF2 #1 are : (forward: 5'-GAT CCC CGG CTC AAG TGG CTT AAC AAG GTT CAA GAG ACC TTG TTA AGC CAC TTG AGC CTT TTT A-3'everse: 5'- AGC TTA AAA AGG CTC AAG TGG CTT AAC AAG GTC TCT TGA ACC TTG TTA AGC CAC TTG

AGC CGG G); pSuper-puro-shIRF2 #2 are : (forward: 5'- GAT CCC CGG CAA TCC ATA CAG GAA AGC ATT CAA GAG ATG CTT TCC TGT ATG GAT TGC CTT TTT A-3', reverse: 5'- AGC TTA AAA AGG CAA TCC ATA CAG GAA AGC ATC TCT TGA ATG CTT TCC TGT ATG GAT TGC CGG G); pSuper-puro-shIRF2 #3 are : (forward: 5'- GAT CCC CGG CTC AAG TGG CTT AAC AAG GTT CAA GAG ACC TTG TTA AGC CAC TTG AGC CTT TTT A-3', reverse: 5'- AGC TTA AAA AGG CTC AAG TGG CTT AAC AAG GTC TCT TGA ACC TTG TTA AGC CAC TTG AGC CGG G).

Cell culture and DNA transfection

HEK293T-APP695 cells are described (Gwon et al., 2012; Mitsuishi, Y. et al, 2010). HEK293T and stable cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, SH30243.01) containing 10% (v/v) fetal bovine serum (Hyclone, SH30919.03), and 50 μg/ml Gentamicin (GIBCO, 15750-060), a 37℃ under an atmosphere of 5% CO2. IRF2 KO Cells were selected with 1mg/ml puromycin. According to the manufacturer's instructions, transfection was performed using lipofector-pMAX transfection reagent (APTABIO) or PEI (Sigma, 764647).

Genome-wide functional screening using cDNA

Genome-wide functional screening was performed as described previously (Han et al, 2014). By modifying pC99-GVP and UAS-Luciferase assay, we generated C99-TetOn and TRE-GFP fusion constructs to regulate its activity with doxycycline. HEK293T cells were co-transfected with pC99-TetOn, pTRE-GFP, pDsRed monomer, and either pCtrl or each cDNA for 24 h and then incubated with conditioned media containing 100 ng/ml doxycycline (Sigma-Aldrich) for another 24 h. Once C99-rtTA was cleaved by γ-secretase, the cleavage product AICD-rtTA was transported into the nucleus and induces GFP expression through TRE-GFP vector in the presence of doxycycline. Compared to control, the putative positive cDNA clones which significantly increased green fluorescence under fluorescence microscope (Olympus) were isolated. After confirming the stimulatory effect on the reporter activity, the putative positive cDNA clones were tested for its stimulatory effect on Aβ generation using sandwich ELISA kit.

***In vitro* AICD generation assay**

In vitro AICD generation assay was conducted as previously described with minor modification (Thesco G et al., 2005). The harvested cells were lysed by sonication in buffer A (50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM 1, 10-phenanthroline monohydrate, 2 mM EDTA and protease inhibitor cocktail). The

homogenate was centrifuged at 1,000 ×g for 10 min and the remaining supernatant was further centrifuged at 10,000 ×g for 15 min. The membrane fraction in pellets was washed once with buffer A and centrifuged again. The final membrane pellet was resuspended with buffer A and total protein was quantified by Bradford assay (Biorad). The same amount of protein was incubated at 37°C for 2 h with or without 2 μM Compound E (Comp. E).

BN-PAGE

BN-PAGE was performed as previously described (Bigl M et al., 1999; Zhong C et al., 2013). The same amount of microsomal membranes protein was solubilized in BN-PAGE buffer (0.5% dodecylmaltoside, 20% glycerol and 25 mM Bis-Tris pH 7.0) for 60 min on ice. After ultracentrifugation at 100,000 ×g for 30 min, the same volume of soluble protein was separated by BN-PAGE at 4°C and transferred into PVDF membrane. The transferred blot was destained for 1 h in destaining solution (distilled water: methanol: acetic acid, 6: 3: 1) and analyzed with Western blotting.

ELISA

Aβ1-40 and Aβ1-42 levels in the culture media were measured by sandwich ELISA kits (Invitrogen and IBL). Each harvested samples were subjected to assay following the manufacturer's instruction.

Luciferase reporter gamma secretase assay

Luciferase reporter γ-secretase activity assay was described in ref (Gwon et al., 2011). HEK293T cells were co-transfected with pC99-GVP, pUAS-Luciferase, pβ-galactosidase and experimental control genes. After 24 h, cell extracts were analyzed by luciferase assay following the manufacturer's instruction (Promega). The luciferase activity was normalized by β-galactosidase activity for transfection efficiency.

SDS-PAGE and western blot analysis

Western blot analysis was performed using standard techniques. Cells were lysed with sample buffer (10% glycerol, 2% SDS, 62.5 mM Tris-HCl, 2% β-mercaptoethanol, pH 6.8). For the preparation of membrane protein, cells were solubilized in 1 % CHAPS buffer containing protease inhibitor cocktail and centrifuged at 10,000 g for 10 min. The soluble supernatants were subjected to

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to polyvinylidene fluoride membrane (ATTO, AE-6667-P) using a Bio-Rad semi-dry transfer unit (Bio-Rad). Immunoblot analysis was then performed and visualized by the enhanced chemiluminescence method.

Statistical analysis

All experiments were performed in triplicate parallel instances and repeated at least three times. Statistical analyses were carried out using the Microsoft Office 2013 Excel software package (Microsoft Corporation). Mean values were compared using unpaired t-tests.

RESULTS

Cell-based functional screening to isolate γ-secretase regulator

To isolate γ-secretase regulator, I have screened cDNA expression library using a cell-based functional screening assay as described previously (Han et al., 2014). Briefly, HEK293T cells were co-transfected with C99-TetOn (a fusion protein containing APP C99 and TetOn), TRE-GFP (green fluorescence protein fused to TatOn-response element), DsRed Monomer (transfection control for normalization) and each cDNA in mammalian expression vector. If the cDNA regulates γ-secretase activity in the transfected cells, the processing of C99-tetOn by γ-secretase would be positively or negatively affected and the cleavage product, TetOn, moves to nucleus and binds to TRE, resulting in a change in GFP signal under green fluorescence microscope. Thus, compared to the negative control, I can isolate putative positive clones that can affect GFP signal reflecting γ-secretase activity (Figure 1A).

In order to confirm this screening assay, I transfected HEK293T cells with C99-TetOn, TRE-GFP, DsRed Monomer and either negative control vector (pcDNA3) or a positive clone GSK3β as described above and examined the green

fluorescence under microscope. Based on measurement of the red fluorescence of each transfection, I confirmed that the transfection efficiency was even among transfections. As expected, I found that compared to the negative control, GFP signal was increased by ectopic expression of the positive clone (GSK3β), confirming that this screening assay works out well to isolate new clones (Figure 1B).

Previous laboratory members screened cDNA expression library and isolated 20 putative positive clones. Using this cell-based functional screening, I screened 20 cDNA expression clones. As a result, I found that 9 clones were effective to increase reporter activity reflecting the activity of γ-secretase (Figure 2). Among them, I found that IRF2, F6 and E11 largely increased GFP signal compared to control and that this increase did not result from different transfection efficiency.

Effects of the positive clones on γ-secretase activity and AICD generation

To further assess that the putative clones actually regulate enzyme activity of γ-secretase, another assay using luciferase reporter system was employed (Gwon et al., 2011). This assay is based on the similar principle with cell-based functional screening (GFP reporter) and measures luciferase reporter activity. When I tested effects of the positive clones, I found that the values of luciferase

activity were significantly increased by overexpression of F6, E11, or interferon regulatory factor 2 (IRF2) (Figure 3A). From both cell-based functional screening and luciferase reporter system, I concluded that F6, E11, and IRF2 clones were highly effective to activate γ-secretase among 9 clones.

In order to prove that the three putative clones increase enzymatic activity of γ-secretase, *in vitro* assay was assessed to directly examine the proteolytic processing of APP. This *in vitro* assay measures the production of APP intracellular domain (AICD), a cleavage product of γ-secretase, from APP in cell extracts. When this assay was carried out, cell extracts harboring overexpressed positive control, was effective to generate AICD and this production of AICD was inhibited by treatment with Compound. E, an inhibitor of γ-secretase. Then, incubation of cell extracts harboring one of these clones also increased AICD generation compared to the control, indicating that F6, E11, and IRF2 clones altogether increase AICD generation by increasing the γ cleavage of APP through γ-secretase. Calnexin (CANX) was used as an internal control for the cleavage assay. These results suggest that F6, E11 and IRF2 clones might increase γ-secretase activity.

IRF2 overexpression increases γ-secretase component and complex level

Then, I attempted to examine how these clones regulate γ-secretase activity. First, I tried to investigate whether or not the expression levels of γ-secretase components were affected. From western blot analysis, I found that ectopic expression of IRF2 increased the levels of γ-secretase components. Especially, the levels of NCT, APH-1aL and PS1-NTF were significantly increased by IRF2 overexpression. In addition, the levels of NCT and APH-1aL seemed to increase by F6 overexpression. On the contrary, the levels of γ-secretase components were not affected by E11 overexpression. (Figure 4).

To further determine whether clones affects the formation of the γ-secretase complex and thereby increases the enzymatic activity, a blue-native polyacrylamide gel electrophoresis (BN-PAGE) assay was used (Bigl M et al., 1999; Zhong C et al., 2013). The crude membrane fraction was separated from cells by centrifugation, and this fraction was lyzed in 1% digitonin-containing lysis buffer. Western blotting following BN-PAGE confirmed that ~ 440 kDa of holo γ-secretase mature protein complex contains NCT, APH-1a, PEN2 and PS1-NTF (Nyabi O et al., 2003). Protein amounts in the crude membrane fraction was quantified by the level of Calnexin.

Then I assessed the effects of the clones on the formation of holo γ-secretase mature protein complex. The results from western blot analysis showed that the amount of a high molecular weight protein complex of around 440 kDa was

apparently increased by ectopic expression of IRF2 (Figure 5). Similar increase of 440 kDa γ-secretase mature protein complex was also found in cells overexpressing E11. On the other hand, there was no change in 440 kDa γ-secretase mature protein complex by F6 overexpression. Taken together, these results suggest that IRF2 and E11 might increase the levels of holo γ-secretase mature protein complex.

IRF2 expression alters $A\beta_{1-42}/A\beta_{1-40}$ ratio

Among these 3 clones, I decided to focus on IRF2 because its effect on γ-secretase was the biggest. To see whether the changes in the amount of γ-secretase complex could affect Aβ production, the levels of secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ were measured by sandwich ELISA. Result from the ELISA showed that the level of $A\beta_{1-42}$ in the medium was increased by 20% following ectopic expression of IRF2 in HEK293 cells stably expressing APP695 (HEK293/APP695), while that of $A\beta_{1-40}$ was reduced (Figure 6). On the other hand, overexpression of F6 or E11 had no change in the production of both $A\beta_{1-40}$ and $A\beta_{1-42}$. Thus, these results indicate that IRF2 regulates Aβ production, leading to change in the ratio of $A\beta_{1-40}/A\beta_{1-42}$.

Knockdown of IRF2 reduces complex level of γ-secretase

As the formation of holo γ-secretase mature protein complex increased due to IRF2 overexpression, knockdown effects of IRF2 expression on the formation of the γ-secretase complex were examined. I reduced IRF2 expression using shRNA. The results from western blot analysis following BN-PAGE assay showed that levels of γ-secretase complex level were decreased in IRF2 knockdown cells compared to control mock cells (Figure 7A). In general, levels of all four core proteins of γ-secretase complex around 440 kDa were reduced in by knockdown of IRF2. In addition, the luciferase reporter assay also showed decreased γ-secretase activity by knockdown of IRF2 in general (Figure. 7B). Together with overexpression analysis, the results suggest that IRF2 regulates levels of holo γ-secretase mature protein complex leading to modulate γ-secrease activity.

IRF2 overexpression reduces NotchΔE processing

Since γ-secretase can process other substrates, such as Notch, I was curious whether the activation of γ-secretase by IRF2 modulated the processing of those. Especially, I addressed Notch cleavage by γ-secretase by determining the generation of Notch intracellular domain (NICD) in HEK293T cell expressing a

truncated form of the Notch receptor tagged with GFP (NotchΔE-GFP) (Kerppola T.K., 2008; Han et al., 2014). As reported, generation of NICD was significantly suppressed by the treatment with Compound. E, inhibitor of γ-secretase. Using this assay, I addressed effect of IRF2 on Notch processing. The results from western blotting revealed that ectopic expression of IRF2 significantly reduced NICD production compared to that of control (Figure 8A). On the other hand, generation of NICD was increased by E11 overexpression and was not affected by F6 expression. Thus, unlike its stimulatory effect on $A\beta_{1-42}$ production, IRF2 overexpression reduces NICD generation.

Ectopic expression of IRF family increases γ-secretase activity

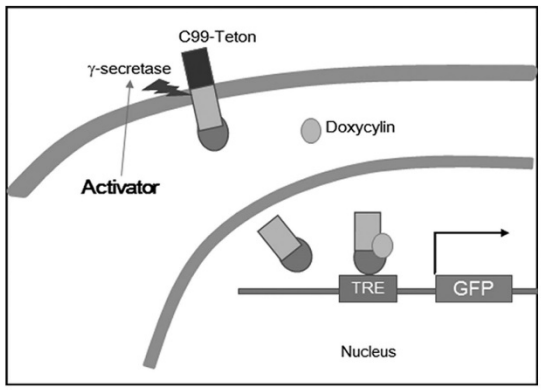
IRE2 is a member of interferon regulatory factor (IRF) family which consists of more than 8 IRFs. Thus, I decided to test whether other members of IRF family could also affect γ-secretase activity using the cell-based functional assay. Interestingly, the activity of γ-secretase was all increased by the expression of IRF1, 2, 3 or 7 compared to that of mock (Figure 9A). In addition, the luciferase reporter assay also showed that γ-secretase activity was also increased by ectopic expression of those IRF family (Figure. 9B). It is interesting to note that other IRF1, 3 and 7 were even better to increase the reporter activity of γ-

secretase than IRF2 did. These results suggested that IRF1, IRF3 and IRF5 as well as IRF2 may increase γ-secretase activity.

Figure 1 │Cell-based assay for measurement of γ-secretase activity.

(A) A schematic image of cell-based functional screening assay for finding γ-secretase activator. (B) Cell-based functional assay. HEK293T cells were co-transfected with pC99-Tet, pTRE-GFP, mRFP and either pcDNA3 (Mock) or GSK3β (+Ctrl) for 6 h and then left untreated (no doxy) or treated with doxycycline (1 μM) for another 12 h. Cells were then observed under fluorescence microscope.

A


C99-Teton
γ-secretase
Doxycylin
Activator
TRE
GFP
Nucleus

B

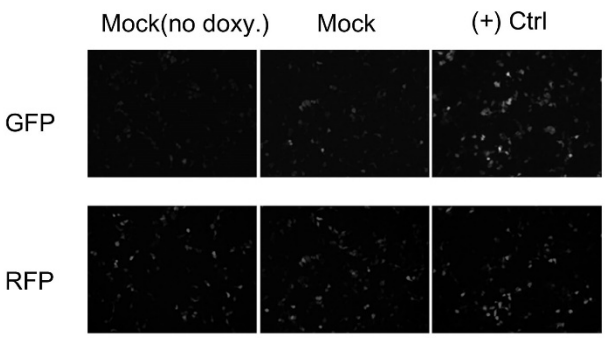

Mock(no doxy.)
Mock
(+) Ctrl
GFP
RFP

Figure 2 | Functional screening of γ-secretase activity regulator.

HEK293T cells were co-transfected with pC99-Tet, pTRE-GFP, mRFP and either pcDNA3 (Mock) or the indicated cDNAs for 6 h and then treated with doxycycline (1 μM) for another 12 h. Cells were then observed under fluorescence microscope. GFP signal: TRE-GFP, RFP signal: mRFP.

<GFP signal>

<RFP signal>

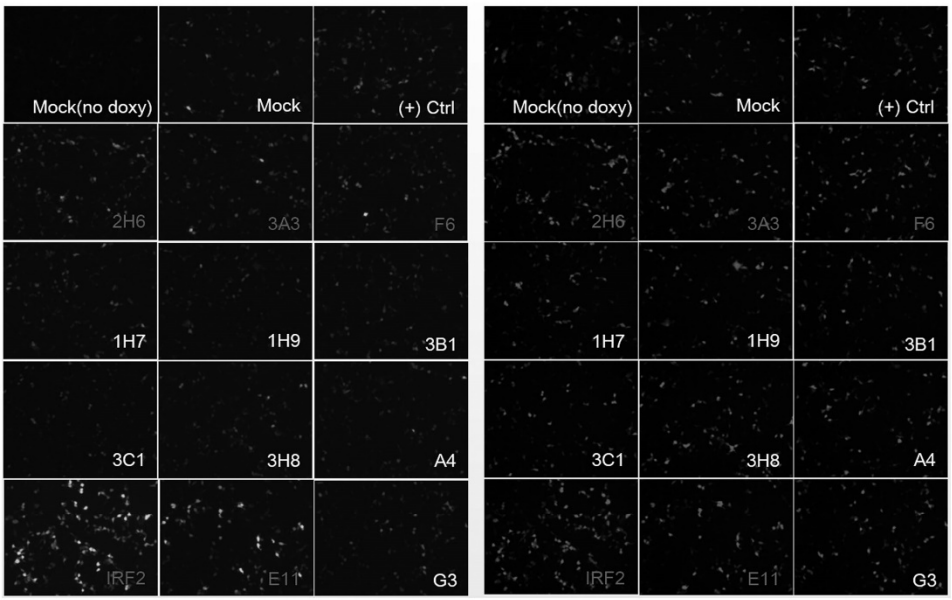
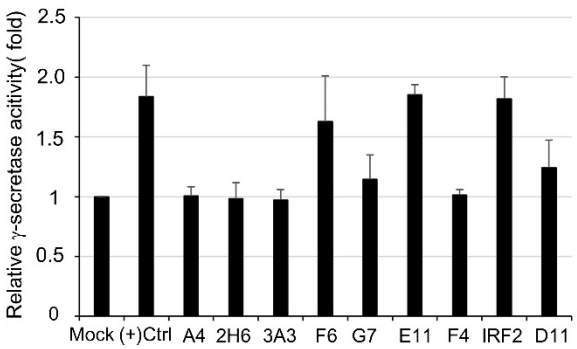


Figure 3 | Effects of the positive clones on γ -secretase activity and AICD generation.

(A) Luciferase reporter assay to monitor the γ-secretase activity. HEK293T cells were co-transfected with pC99-GVP, pUAS-Luciferase, pβ-galactosidase, mRFP and either pcDNA3 (Mock) or one of the positive clones. After 24 h, cell extracts were prepared and analyzed for the luciferase activity. The luciferase activity was normalized by that of β-galactosidase and is expressed as -fold activation relative to the control. Bars represent mean ± SD (n = 3). (B) Selected clones stimulate AICD generation. HEK 2933T/APP695 cells were transfected with either p3xflag CMV14 (Mock), pIRF2-flag (IRF2), pF6-flag (F6) or pE11-flag (E11). After 48 h, crude membrane fraction was prepared by centrifugation. The membrane fraction was then incubated at 37℃ for 3 h with or without 2 μM Compound. E (Comp. E) as a negative control. The harvested sample was separated by SDS-PAGE for western blotting. The level of AICD on the blot was measured by densitometric analysis and their relative ratio to control was represented at the bottom after normalization by CANX.

A


Relative γ-secretase acitivity(fold)
2.5
2.0
1.5
1
0.5
0
Mock (+)Ctrl A4 2H6 3A3 F6 G7 E11 F4 IRF2 D11

B

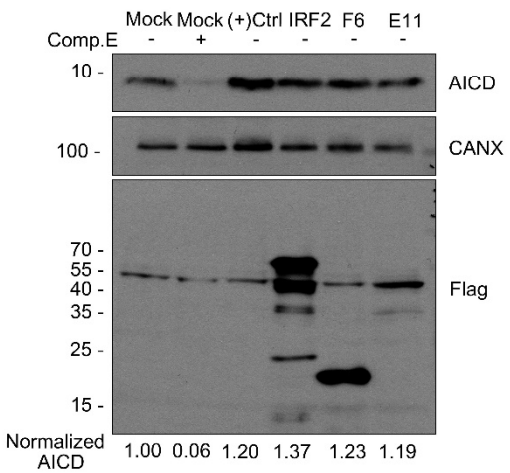
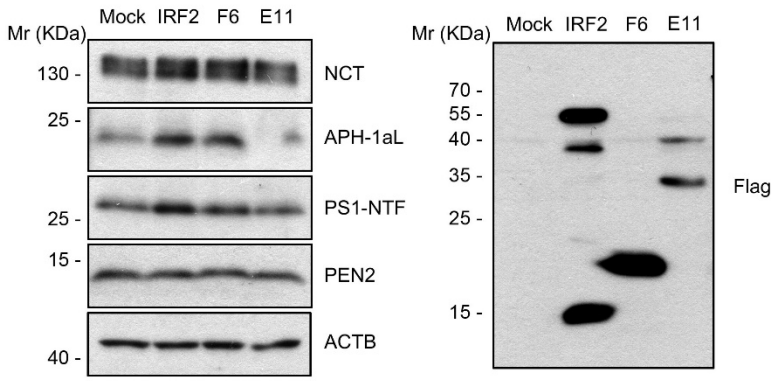

Mock Mock (+)Ctrl IRF2 F6 E11
Comp.E - + - - - -
10 -
AICD
100 -
CANX
70 -
55 -
40 -
35 -
25 -
15 -
Flag
Normalized AICD
1.00 0.06 1.20 1.37 1.23 1.19

Figure 4 | Effects of IRF2 overexpression on the component levels of γ-secretase complex.

(A, B) Overexpression of IRF2 increases the level of γ-secretase components. HEK293T cells were transfected with p3xflag CMV14 (Mock), pIRF2-flag (IRF2), pF6-flag (F6) or pE11-flag (E11) for 48 h. and then solubilized in 1 % CHAPS lysis buffer. The lysates were analyzed by western blotting using the indicated antibodies against γ-secretase components (left) or the positive clones (Flag, left) (A). Quantification of the signals on the blots was performed with densitometry analysis (B)

A


Mock IRF2 F6 E11
Mr (KDa)
130 -
25 -
25 -
15 -
40 -
NCT
APH-1aL
PS1-NTF
PEN2
ACTB
Mr (KDa)
Mock IRF2 F6 E11
70 -
55 -
40 -
35 -
25 -
15 -
Flag

B

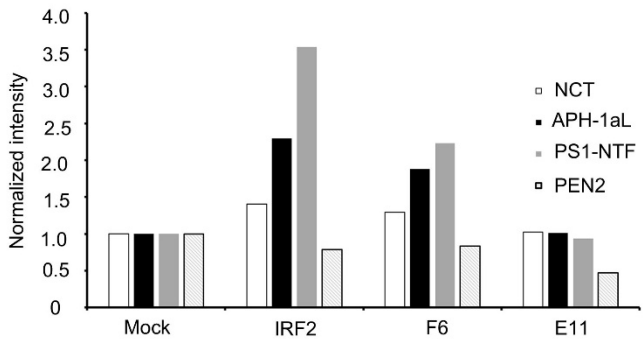

Normalized intensity
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Mock
IRF2
F6
E11
NCT
APH-1aL
PS1-NTF
PEN2

Figure 5 | Stimulatory effects of IRF2 overexpression on γ-secretase complex level.

IRF2 overexpression increases the amount of γ-secretase complex on the blue-native (BN) gel. After transfection of HEK293T cells with p3xflag CMV14(Mock), pIRF2-flag(IRF2), pF6-flag(F6) or pE11-flag(E11) for 48 h, crude membrane fractions was prepared after solubilization with 1 % digitonin, separated by BN-PAGE, and analyzed by western blotting using antibodies against APH-1aL, NCT, PEN2, PS1-NTF (upper and middle). Whole cell lysates were examined by western blotting (CANX, lower).

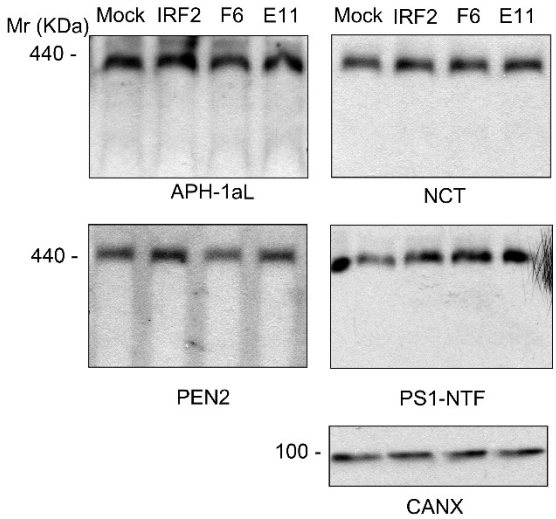

Mr (KDa)
Mock IRF2 F6 E11
Mock IRF2 F6 E11
440 -
APH-1aL
NCT
440 -
PEN2
PS1-NTF
100 -
CANX

Figure 6 | IRF2 expression alters $A\beta_{1-42}/A\beta_{1-40}$ ratio.

HEK 293T/APP695 cells were transfected with either p3xflag CMV14 (Mock), pIRF2-flag (IRF2), pF6-flag (F6) or pE11-flag (E11) and maintained in the conditioned media for 24 h. The levels of Aβ1–40 and Aβ1–42 in the media was then measured using ELISA kit. Bars represents mean ± SD (n = 3)

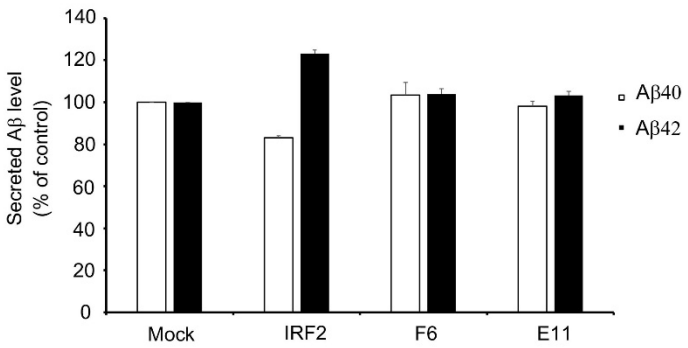
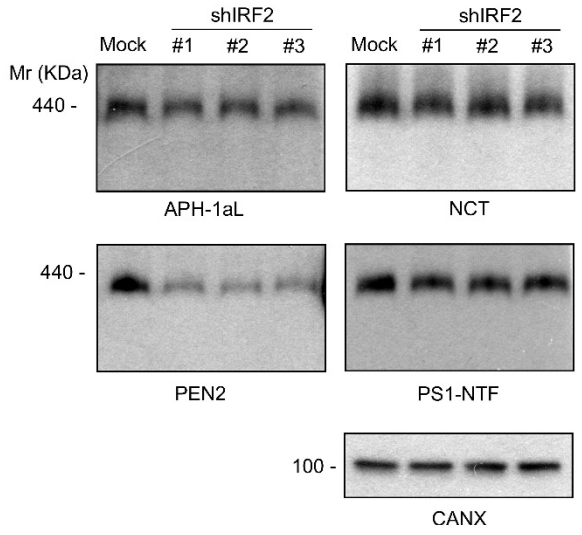

140
120
100
80
60
40
20
0
Secreted Aβ level
(% of control)
Mock
IRF2
F6
E11
Aβ40
Aβ42

Figure 7 | Knockdown of IRF2 reduces γ-secretase complex level.

(A) Knockdown of IRF2 reduces the amount of γ-secretase complex on the blue-native (BN) gel. After transfection of HEK293T cells with pSuper-puro (Mock), pshIRF2 #1 (#1), pshIRF2 #2 (#2) or pshIRF2 #3 (#3) for 48 h, crude membrane fractions was prepared after solubilization with 1 % digitonin, separated by BN-PAGE, and analyzed by western blotting using antibodies against APH-1aL, NCT, PEN2, PS1-NTF (upper and middle). Whole cell lysates were examined by western blotting (CANX, lower). (B) Luciferase reporter assay to monitor the γ-secretase activity. HEK293T cells were co-transfected with pC99-GVP, pUAS-Luciferase, pβ-galactosidase, mRFP and either pSuper-puro (Mock), pshIRF2 #1 (#1), pshIRF2 #2 (#2) or pshIRF2 #3 (#3). After 48 h, cell extracts were prepared and analyzed for the luciferase activity. The luciferase activity was normalized by that of β-galactosidase and was expressed as -fold activation relative to the control. Bars represent mean ± SD (n = 3).

A


shIRF2
shIRF2
Mock
#1
#2
#3
Mock
#1
#2
#3
Mr (KDa)
440 -
APH-1aL
NCT
440 -
PEN2
PS1-NTF
100 -
CANX

B

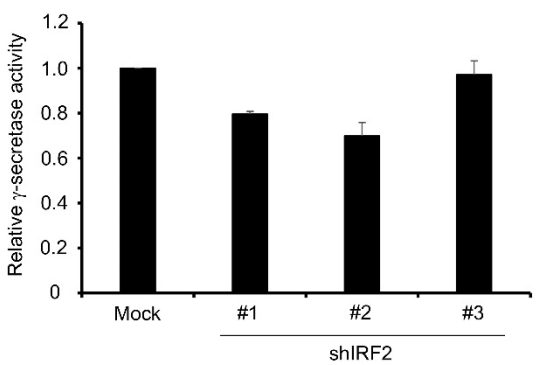
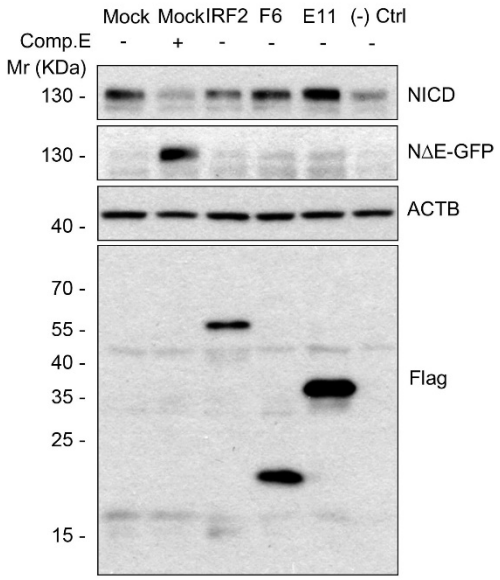

Relative γ-secretase activity
1.2
1.0
0.8
0.6
0.4
0.2
0
Mock
#1
#2
#3
shIRF2

Figure 8 ｜IRF2 overexpression reduces NotchΔE processing.

(A, B) IRF2 overexpression decreases the cleavage of Notch. HEK293T cells were transfected with pNotchΔE-GFP (NΔE-GFP) and either pcNDA3, pIRF2-flag, pF6-flag or pE11-flag for 24 h and cell extracts were analyzed with western blotting (A). Quantification of the signals on the blots was performed by densitometry analysis (B). Bars represents mean ± SD (n = 3)

A


Mock
Mock
IRF2
F6
E11
(-) Ctrl
Comp.E
-
+
-
-
-
-
Mr (KDa)
130 -
NICD
130 -
NΔE-GFP
ACTB
40 -
70 -
55 -
40 -
35 -
25 -
Flag
15 -

B

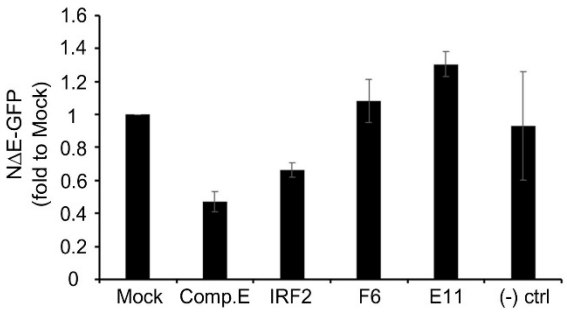
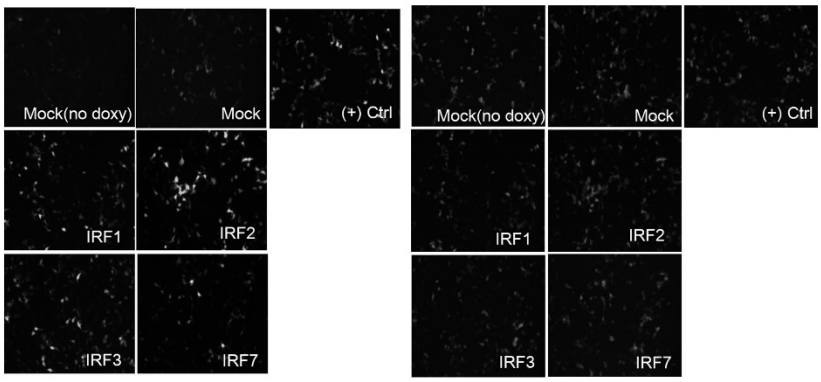

NΔE-GFP
(fold to Mock)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mock
Comp.E
IRF2
F6
E11
(-) ctrl

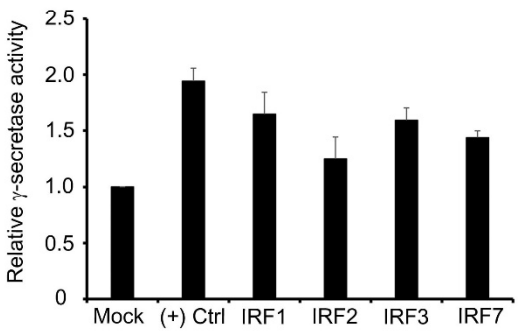
Figure 9 │ Ectopic expression of IRF family increases γ-secretase activity.

(A) Cell-based assay of IRF family. HEK293T cells were co-transfected with pC99-Tet, pTRE-GFP, mRFP and pcDNA3 (Mock), IRF1, IRF2, IRF3 or IRF7 for 6 h, and treated with doxycycline (1 μM) for another 12 h. GFP fluorescence were observed under fluorescence microscope. (B) Luciferase reporter assay to monitor the γ-secretase activity. HEK293T cells were co-transfected with pC99-GVP, pUAS-Luciferase, pβ-galactosidase, mRFP and pcDNA3 (Mock), IRF1, IRF2, IRF3 or IRF7. After 24 h, cell extracts were analyzed for luciferase activity. The luciferase activity was expressed as -fold activation relative to the control. Bars represent mean ± SD (n = 3).

A


Mock(no doxy)
Mock
(+) Ctrl
IRF1
IRF2
IRF3
IRF7
Mock(no doxy)
Mock
(+) Ctrl
IRF1
IRF2
IRF3
IRF7

B


Relative γ-secretase activity
2.5
2.0
1.5
1.0
0.5
0
Mock
(+) Ctrl
IRF1
IRF2
IRF3
IRF7

DISCUSSION

In this study, we identified IRF2 as a novel activator of γ-secretase and Aβ generation through a gain-of-function (GOFS) screening procedure, including C99 cleavage-induced changes in GFP intensity in cells, which was followed by a secondary screening process that measured γ-secretase activity using a C99-GVP/UAS-luciferase reporter and measurement of Aβ levels using ELISA. Among putative positive clones, IRF2 was identified as being the most effective in all three assays. In the previous study, I identified OCIAD2 and DUSP26 as a novel γ-secretase activator with the same approach utilizing cDNA expression library (Han et al., 2014; Jung et al., 2016). This approach was selected as a screening approach using a cDNA expression library over a loss-of-function screen (LOFS) of a siRNA library because the objective was to identify an activator of γ-secretase and a gain of function may not have always been detected in the cell-based assays of a LOFS under certain conditions (Han et al., 2014). Thus, GOFS has unique advantage over LOFS in the circumstance in which the signal is not active.

IRF2 is a transcription factor enriched in the nucleus and is expressed mainly

in the brain, kidney, and lung (Uhlén et al., 2015; Human Protein Atlas). Among IRF family, it was surprising to know that IRF1, 3 and 7 all increase γ-secretase activity as much as IRF2. In general, the expression of IRF1 is induced by the treatment with interferon-gamma (IFN-γ), type II interferon. On the contrary, the expression of IRF2 is known to be constitutive in most cases, while IRF2 expression can also be increased by type I interferon signaling like interferon-alpha/beta (IFN-α/IFN-β), even if it is weaker than IRF1 (Harada et al., 1989). Besides, it was shown that the treatment with IFN-γ can also induce up-regulation of IRF2 in human esophageal cancer cells (Wang et al., 2008). Therefore, it is necessary to examine the expressional variation of IRF family in the brain of AD patients and compare those with age-matched controls.

Unlike conventional concept about little function of immune system in AD, recently, genetic and preclinical data have shown that activation of immune system accompanies AD pathology and contributes to the pathogenesis of this disease by discovery of mutation genes encoding triggering receptor expressed on myeloid cells (TREM2) (Jonsson et al., 2013) and myeloid cell surface antigen (CD33) (Bradshaw et al., 2013). Especially, it was already shown that levels of inflammatory cytokines, chemokines and other immune mediators are increased in the tissues and body fluids of individuals with AD or prodromal forms of this disease (Heppner et al., 2015). In this context, IRF family might be important as

an element linking IFN pathway to AD pathogenesis through regulation of γ-secretase activity.

A notion that the $A\beta_{1-42}/A\beta_{1-40}$ ratio was altered by IRF2 expression is interesting. Alteration of $A\beta_{1-42}/A\beta_{1-40}$ ratio is a well-known a pathogenic factor observed in the Presenilin mutants of familial type AD causing increase of $A\beta_{1-42}/A\beta_{1-40}$ ratio and leading to aggregate prone status (Mucke., 2011). In particular, these mutations change the biochemical character of γ-secretase and alters the interaction with C99. From this point of view, not only increase in the level of γ-secretase but something other event, such as change in the interaction with C99 should be examined. It thus remains to further address a question on how IRF2 alters $A\beta_{1-42}/A\beta_{1-40}$ ratio in molecular level.

Another important finding is that unlike C99 cleavage, Notch processing is inhibited by IRF2 overexpression. This point of regulation cannot be explained only by the increase of γ-secretase complex and activity. One of possibilities on why the selective processing of γ-secretase substrate occurs by ectopic expression of IRF2 is that other unidentified regulator might work together with γ-secretase core complex to provide the selectivity of γ-secretase substrate following the expression of IRF2. It is also possible that IRF2 expression affects subcellular localization or modification of γ-secretase complex for substrate specificity.

In conclusion, I conducted a cell-based functional screening to find a novel γ-secretase regulator and found IRF2 that alters the $A\beta_{1-42}/A\beta_{1-40}$ ratio with Notch-sparing activity.

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국문초록

알츠하이머병은 인지능력 저하와 기억력의 상실을 동반하는 대표적인 퇴행성 신경질환이다. 알츠하이머병의 주요한 특징은 아밀로이드 베타 (amyloid beta) 단백질이 축적되어 생기는 아밀로이드 플라크 (plaque)와 타우 단백질이 과인산화 되어 형성되는 신경섬유매듭 (neurofibrillary tangle)이 각각 환자 두뇌의 신경세포 안과 밖에서 관찰되는 것이다. 아밀로이드 베타 단백질은 베타 세크리테이즈와 감마 세크리테이즈가 아밀로이드 베타 전구단백질을 연속적으로 절단함으로써 생성된다. 감마 세크리테이즈는 4가지 단백질이 결합하여 형성되는 단백질 복합체로서 감마 세크리테이즈에 의한 절단은 아밀로이드 베타의 생성효율을 조절하는 매우 중요한 단계이다. 그럼에도 불구하고 감마 세크리테이즈를 조절할 수 있는 분자적 기전은 현재까지 밝혀진 바가 많지 않다. 따라서 이 논문에서는 감마 세크리테이즈를 활성화시킬 수 있는 유전자를 동정하여 IRF2를 발굴하였으며, 기능적 연구를 수행하였다.

IRF2는 핵에 존재하는 전사인자로 알려져 있으며, 주로 면역반응 관련 인자의 발현조절에 관여한다고 알려져 있다. IRF2가 세포에서 발현되었을 때 뭉치기 쉬운 형태의 아밀로이드 베타의 생성이 증가됨을

확인하였고, 감마 세크리테이즈의 활성이 높아졌음을 확인하였다. 또한 세포 내 실험을 통해 IRF2는 감마 세크리테이즈를 구성하는 단백질의 양을 증가시켜 활성화된 감마 세크리테이즈의 양을 늘린다는 것을 발견하였다. 여기에 더해 세포 내에서 shRNA system 을 통해 IRF2의 발현을 감소시킨 결과, 활성화된 감마 세크리테이즈의 양 및 활성이 감소하는 것을 관찰함으로써 추가적으로 IRF2가 감마 세크리테이즈 활성을 조절하는 데 중요함을 밝혔다. 또한 IRF2는 아밀로이드 베타 전구단백질의 절단은 촉진시키지만, 감마 세크리테이즈의 또 다른 기질로 알려진 Notch 단백질의 절단은 감소시키는 것으로 관찰되어 IRF2가 선택적으로 감마세크리테이즈의 활성을 조절할 수 있음을 확인하였다. 그리고 다른 IRF family 또한 감마세크리테이즈의 활성을 증가시키는 것을 관찰함으로써 IRF family 가 감마 세크리테이즈 조절에 역할이 있음을 확인하였다.

이상의 결과들을 종합하여 이 논문에서는 IRF2 가 감마 세크리테이즈를 조절함으로써 아밀로이드 베타 생성을 증가시키는 감마 세크리테이즈 조절 유전자라는 것을 제안한다.

주요어: 알츠하이머병, 아밀로이드 베타, 감마 세크리테이즈, IRF2

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