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学 位 論 文

Investigating the role of ECRG4 in Alzheimer's
disease pathology and diagnosis
(アルツハイマー病の病理と診断における
ECRG4 の役割の調査)

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1. List of publications and presentations

Publications:

1. Lifu Zhang, Naoki Ikeda, Shuji Takeda, Naoto Oikawa, Shigeo Murayama, Uichi Koshimizu, Ichiro Yabe & Toru Kondo
Plasma ECRG4 as a novel diagnostic marker for Alzheimer's disease associated with oligodendrocyte dysfunction
American Journal of Translational Research, (2026)
2. Naoto Oikawa, Lifu Zhang, Toru Kondo
Fragment of esophageal cancer-related gene-4 induces lysosome-dependent cell death via cathepsin secretion in astrocytes
Tissue and Cell, (2026)

Presentations:

2. Abstract

Background and Purpose:

Alzheimer's disease (AD) is a subtype of dementia that leads to problems with memory, thinking, and behavior. It is a global health epidemic and a leading cause of morbidity and mortality among the elderly (Tarawneh and Holtzman, 2012). According to estimates by the Alzheimer's Association, approximately 7 million people in the United States currently have AD, and the global number of affected individuals exceeds 32 million (Alzheimer's & Dementia, 2023, Gustavsson et al., 2023).

AD is a progressive neurodegenerative disease associated with specific pathological changes, including the formation of cytotoxic amyloid- β (A β) plaques, intracellular neurofibrillary tangles (NFTs) caused by hyperphosphorylated tau protein, and neurovascular dysfunction (Bertram et al., 2010, Querfurth and LaFerla, 2010a, Zlokovic, 2011). The accumulation of A β is considered a key driver of AD, making it critical to elucidate the molecular mechanisms underlying A β plaque formation in the brain (Jack and Holtzman, 2013). Currently, therapeutic Antibodies (Abs) have been developed to eliminate these plaques (Logovinsky et al., 2016, Rashad et al., 2022, Sevigny et al., 2016) and have been approved by the Food and Drug Administration. However, the therapeutic effects of these Abs are limited to patients with the mild cognitive impairment (MCI) or early-stage AD (EAD), creating a pressing demand for the development of new diagnostic methods that can identify MCI and EAD patients with convenience and high sensitivity.

Esophageal cancer-related gene 4 (ECRG4), also known as AUGURIN, was thought to be a tumor suppressor gene, as studies showed its expression was significantly reduced in malignant tumors compared to the surrounding normal tissues (Gotze et al., 2009, Yue et al., 2003). Extensive evidence demonstrated that overexpression of ECRG4 not only inhibits the proliferation and migration of various cancer cells but also induces cell death, although its molecular mechanisms remain unclear (Kujuro et al., 2010). It was also shown that ECRG4 not only induces the expression of pro-inflammatory factors such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in macrophages and microglia but also plays a role in brain aging by inhibiting neural stem cell proliferation (Kujuro et al., 2010). Additionally, elevated levels of ECRG4 have been observed in the brains of AD model mice and AD patients (Chung et al., 2018, Woo et al., 2010). These findings suggested that ECRG4 serves as a novel diagnostic marker for detecting AD.

Currently, several methods are available for diagnosing AD, such as amyloid- β positron emission tomography (A β PET), cerebrospinal fluid (CSF) measurements of A β , and mass spectrometry-based detection of A β in plasma (Harada et al., 2018). However, PET and mass spectrometry require specialized laboratory equipments and collecting CSF is highly invasive. Therefore, there is a need to develop simpler and less invasive diagnostic methods. Studies have shown that ECRG4 is released or secreted from expressing cells by proteases, such as furin and thrombin (Dang et al., 2012, Gonzalez et al., 2011). My research aims to develop a new enzyme-linked immunosorbent assay (ELISA) system and examine the ECRG4 levels in the plasma and CSF of dementia and non-dementia patients with neurological disorders. I also aim to produce new monoclonal Abs (mAbs) to improve the ELISA system.

Subjects and Methods:

To develop an ELISA system for detecting ECRG4 in body fluids of the patients, both monoclonal and polyclonal Abs against ECRG4 were generated. RNA sequencing (RNA-seq) of the patients' plasma was performed for analyzing the expression profiles. The RNA-seq data was validated using the quantitative reverse-transcription PCR. Cells expressing the candidate genes *in vivo* were selected by Human Protein Atlas. *In vitro* assays, including 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay and immunocytochemistry, assessed ECRG4's effects on the candidate cells. *In vivo* assays, immunohistochemistry was conducted to investigate the ECRG4's effects in brains of mice intracerebrally injected with the peptide. I also examined cellular changes in the hippocampus of AD patients at different stages.

Result:

I developed an ELISA system for detecting ECRG4 using the high-sensitivity mAb 2A8 in combination with the polyclonal antibody OB1246. I observed that ECRG4 increased in the plasma of AD, MCI and dementia groups. RNA-seq analysis using the ECRG4-positive and -negative plasma suggested the adverse effects to neurons and oligodendrocyte (OLG) lineage cells in the ECRG4-positive patients. *In vitro* experiments revealed that ECRG4 induced death in OLGs. ECRG4 injection into the mouse brain caused the reduced intensity of OLG markers, the proliferation of oligodendrocyte progenitor cells (OPCs), their perivascular accumulation, and blood vessels (BVs) rupture. Additionally, perivascular accumulation of OPCs was observed in the hippocampus of EAD patients.

Discussion:

In this study, I developed a sensitive and specific ELISA system to detect ECRG4. Using this system, I found the increased level of ECRG4 in the plasma of patients with AD, dementia, and MCI, compared to non-dementia patients. On the other hand, no significant changes were observed in the CSF of either dementia or non-dementia patients. These data suggested ECRG4 specifically leaked out into blood in MCI and AD patients. Mechanical analysis of the leakage of ECRG4 into the BVs revealed that ECRG4-dependent cytotoxicity to OLG caused OPC clustering-dependent disruption of blood-brain-barrier (BBB). However, it is still needed to unveil the molecular mechanism in detail by identifying the receptor involved in this phenomenon.

One of remaining questions is why ECRG4 increases in the plasma of AD patients but not in their CSF, although it is predominantly expressed in the ventricular zone and choroid plexus (Gonzalez et al., 2011, Nakatani et al., 2019). Aging and neurological disorders may induce ECRG4 expression in the neurons and glial cells as previously shown (Kujuro et al., 2010). Therefore, it is of interest to reveal which cells express ECRG4 and how its expression mechanism is regulated in the cells. Beside the mechanism of ECRG4 expression, ECRG4 may exacerbate AD progression by inducing the expression of inflammatory factors, such as TNF- α and IL6, in microglia and macrophages (Podvin et al., 2015, Moriguchi et al., 2016). These experiments apparently lead to develop novel therapeutic methods for AD.

I established new ECRG4 ELISA system detecting peptides containing amino-acid (AA) 107-132. Since ECRG4 is thought to cleave into several peptides, each of which may exert different functions (Mirabeau et al., 2007), it is important not only to determine their functions in physiological and pathological condition, including AD but also to establish other ELISA systems for detecting each peptide. It is also needed to validate this result by employing larger and more diverse cohorts.

Conclusion:

In conclusion, my study suggests that elevated ECRG4 levels in the plasma serve as a new biomarker for detecting MCI and EAD patients and that ECRG4 plays a critical role in the pathophysiological processes of AD by promoting OLG death and compromising the integrity of the BBB. Further studies need to determine the mechanism of ECRG4 in the AD pathogenesis and develop new therapeutic methods for the disease in the future.

3. List of Abbreviations

AA	Amino acid
Ab	Antibody
A β	Amyloid β
A β PET	Amyloid-beta positron emission tomography
AD	Alzheimer's disease
ANOVA	One-way analysis of variance
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BBB	Blood-brain-barrier
BCA	Bicinchoninic acid
BV	Blood vessel
CC	Corpus callosum
cDNA	Complementary DNA
CERAD-NAB	Consortium to Establish a Registry for Alzheimer's Disease Neuropsychological Assessment Battery
CHST3	Carbohydrate sulfotransferase
CNS	Central nervous system
CSF	Cerebrospinal fluid
DMSO	Dimethyl sulfoxide
EAD	Early-stage AD
ECL	Enhanced chemiluminescence
ECRG4	Esophageal cancer-related gene 4
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ExV-IgG	Extravascular mouse IgG
FCS	Fetal calf serum
GalC	Galactocerebroside
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
HAT	Hypoxanthine-aminopterin-thymidine

HC	Hippocampus
HRP	Horseradish peroxidase
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IL-6	Interleukin 6
KLH	Keyhole limpet haemocyanin
LOX1	Lectin-type oxidized LDL receptor 1
mAb	Monoclonal antibody
MCI	Mild cognitive impairment
MMP9	Matrix metalloproteinase 9
MMSE	Mini mental state examination
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSA	Multiple system atrophy
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NF- κ B	Nuclear factor kappa B
NFL	Neurofilament light chain
NFT	Neurofibrillary tangle
NG2	Neural/glial antigen 2
NOD/SCID	Nod-obese diabetic/ Severe combined immune-deficiency
NP	Neuritic plaque
NPH	Normal pressure hydrocephalus
OLG	Oligodendrocyte
OPC	Oligodendrocyte precursor cell
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDGFR α	Platelet-derived growth factor receptor alpha
PEG	Polyethylene glycol
PET	positron emission tomography
pTau	Phosphorylated tau
qPCR	Quantitative PCR
RIPA	Radio-immunoprecipitation assay
RNA-seq	RNA sequencing
RT	Room temperature

SCX	Strong cation exchange
SD	Standard deviation
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SLC46A3	Solute carrier family 46 member 3
SNP	Single-nucleotide polymorphism
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF α	Tumor necrosis factor alpha
TTC39A	Tetratricopeptide repeat domain 39A
UEA1	Ulex europaeus agglutinin 1
VZ/SVZ	Ventricular zone/Subventricular zone
WB	Western blotting
Wif1	Wnt inhibitory factor 1

4. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative condition marked by specific pathological changes, including the formation of cytotoxic amyloid β ($A\beta$) plaques, intracellular neurofibrillary tangles resulting from hyperphosphorylated tau protein, and neurovascular dysfunction (Bertram et al., 2010, Querfurth and LaFerla, 2010b, Zlokovic, 2011). $A\beta$ is a short peptide derived from the cleavage of amyloid precursor protein (APP), which is processed by enzymes, primarily β - and γ -secretases, to produce $A\beta$ fragments prone to aggregation (Chen et al., 2017). These fragments readily form insoluble fibrillar structures that deposit as plaques, beginning to accumulate in the brain during the early stages of AD (Ow and Dunstan, 2014). This accumulation leads to neuroinflammation, cytotoxicity, and neuronal damage, highlighting the importance of understanding the molecular mechanisms underlying plaque formation (Jack and Holtzman, 2013). Currently, three therapeutic antibodies—Aducanumab, Lecanemab, and Donanemab—have been developed to target $A\beta$, specifically the plaques formed in the brain, and have been approved by the Food and Drug Administration (Logovinsky et al., 2016, Rashad et al., 2022, Sevigny et al., 2016). These antibodies aim to reduce neuroinflammation and neuronal damage by recognizing and binding to $A\beta$ plaques, thereby promoting the clearance of these abnormal protein deposits that accumulate in EAD. However, the therapeutic effects of these antibodies are limited to patients with MCI or EAD, creating a pressing need for the development of new diagnostic methods that can identify MCI and EAD patients with both convenience and high sensitivity.

The diagnostic methods for AD primarily include imaging techniques, such as positron emission tomography (PET) scans and magnetic resonance imaging (MRI), as well as biomarker detection methods, including plasma $A\beta$, tau protein, glial fibrillary acidic protein (GFAP), neurofilament light chain (NFL), and phosphorylated tau (pTau) (Bomasang-Layno and Bronsther, 2021, Fang et al., 2024, Ingannato et al., 2024). Imaging techniques are advantageous for providing early diagnostic information, particularly in revealing structural and functional changes in the brain. In contrast, biomarker detection helps evaluate the disease's pathological processes (Bomasang-Layno and Bronsther, 2021). However, these methods have certain limitations. Imaging techniques can be prohibitively expensive and require specialized equipment, while biomarker detection may suffer from insufficient sensitivity and specificity, which can be influenced by individual variability. Overall, existing diagnostic technologies face challenges in improving accuracy, reducing costs, and enhancing

accessibility (Klyucherev et al., 2022). Therefore, I aim to develop a novel method to detect EAD with high sensitivity, low cost, and a non-invasive approach.

ECRG4, also known as AUGURIN, is encoded by four exons located at the c2orf40 locus on chromosome 2 (Mirabeau et al., 2007). It is thought to be a secreted protein, typically released through the classical endocrine pathway, known as the endoplasmic reticulum-Golgi pathway. However, the exact secretion mechanism of ECRG4 remains unclear (Matsuzaki et al., 2012, Qin and Zhang, 2019, Porzionato et al., 2015). After secretion, ECRG4 is likely cleaved by proteases such as furin and thrombin (Gallwitz et al., 2012, Mirabeau et al., 2007, Ozawa et al., 2011). Further studies are needed to determine which proteases are involved in ECRG4 processing.

Initially, ECRG4 was identified as a tumor suppressor gene, with its expression reduced in malignant tumors compared to surrounding normal tissues, likely due to DNA methylation in the promoter region (Gotze et al., 2009, Yue et al., 2003). ECRG4 overexpression has been shown to inhibit cell proliferation and migration and induce cell cycle arrest in various types of tumor cells (Li et al., 2009). Additionally, it has been suggested that ECRG4 can stimulate the expression of pro-inflammatory factors, such as IL-6 and TNF- α , in macrophages and microglia, potentially contributing to brain aging by inhibiting neural stem cell proliferation (Baird et al., 2012, Kujuro et al., 2010, Lee et al., 2015, Moriguchi et al., 2016).

Recent studies have shown that elevated levels of ECRG4 have been observed in both the brains of AD model mice and the hippocampus of AD patients. Chung et al. demonstrated a significant association between a single-nucleotide polymorphism (SNP) of the *Ecrg4* gene and the joint model of AD pathological features, specifically neuritic plaque (NP) and neurofibrillary tangles (NFT) (Chung et al., 2018). Podvin et al. found that microglia and intravascular monocytes were positive for ECRG4 in the white matter regions of the brains of AD patients (Podvin et al., 2016). Xu et al. also reported that ECRG4 levels were higher in the hippocampus of patients with APOE 3/4 genotypes compared to those with APOE 3/3 non-AD tissues (Xu et al., 2007). These findings suggest a potential link between ECRG4 and AD pathology. Taken together, I hypothesize that the increase in ECRG4 plays a role in the progression of AD by causing inflammation, leading me to examine whether ECRG4 is a novel diagnostic biomarker for AD.

The enzyme-linked immunosorbent assay (ELISA) is a sensitive and widely used immunodetection technique. Its detection principle relies on the specific binding between antigens and antibodies, where enzyme-labeled antibodies amplify and enable signal detection, allowing for the quantitative measurement of biomolecule concentrations such as

proteins, peptides, and hormones (Thermofisher). Due to its high sensitivity and adaptability, ELISA is extensively employed in biomedical research and clinical diagnostics for various molecular analyses, including the detection of AD-associated proteins (Ma et al., 2016, Shinohara et al., 2021). Since ECRG4 can be released or secreted from expressing cells by proteases, including thrombin (Dang et al., 2012, Gonzalez et al., 2011), my research aims to develop a novel ELISA system to detect ECRG4 in body fluids and to investigate whether ECRG4 serves as a diagnostic marker for AD.

5. Materials and Methods

Chemicals and reagents

All chemicals were purchased from Nacalai Tesque and Sigma-Aldrich, unless otherwise indicated.

Mouse and rabbit anti-ECRG4 Abs

The rabbit polyclonal anti-ECRG4 antibody OB1264 was produced by immunizing a rabbit with a synthetic peptide (107-CRNRNGHDYYGDYYQRHYDEDAAGPH-132) by Medical & Biological Laboratories, Japan (Fig. 1). Briefly, injecting an antigen (immunogen) into animals induces them to produce and secrete high levels of antibodies (Abs) into the bloodstream. Several months after repeated immunization, blood (plasma or serum) is collected, and the Abs are purified. The Abs generated through this method are referred to as polyclonal antibodies because they are derived from different B cell clones, resulting in an antiserum that contains numerous Abs reacting to the injected immunogen (MBL).

The mouse monoclonal anti-ECRG4 Abs were produced by immunizing mice with the following synthetic peptides: EDDITYWLN, GDYYQRHYD, PYGFRH, and DDY, respectively, using a standard method (Fig. 1). In brief, a single antibody (monoclonal antibody, mAb) can be stably produced if a single B cell that produces the antibody is isolated and cultured indefinitely. This is achieved by artificially fusing the Ab-producing B cells with immortalized cancer cells (myeloma) to generate hybridomas that can live indefinitely and contain genes encoding specific Abs. Hybridoma clones that produce the desired mAbs with high affinity and specificity are then selected. Typically, it takes 4-6 months from the immunization of animals to the production of mAbs (MBL).

Five million cells from selected hybridomas, including 3B4, 2A8, 1H4, and 1A3, were injected intraperitoneally into 8-week-old Nod-obese diabetic/ Severe combined immune-deficiency (NOD/SCID) mice, and the ascites were subsequently collected. Both mouse and rabbit anti-Ecrg4 Abs were purified using protein A sepharose 4 fast flow, according to the supplier's instructions (GE Healthcare).

ECRG4 sandwich ELISA assay

Ninety-six-well plates (Falcon) were coated with 50 µl of mouse anti-ECRG4 Ab in carbonic acid buffer (0.05 M, pH 9.5) at 4 °C O/N. After washing the plates five times with

washing buffer (0.05% Tween-20/Phosphate-buffered saline [PBS]), the plates were incubated with 150 μ l of blocking buffer (5% skim milk in carbonic acid buffer) for 90 minutes at room temperature (RT). Following three washes with the washing buffer, 50 μ l of samples, including ECRG4 peptide (71-148, AUGURIN precursor, 71-132, prepro-AUGURIN as positive controls at 1 ng/mL) (Phoenix Pharmaceuticals), plasma (1:2), and cerebrospinal fluid (CSF) (1:2), all diluted with assay buffer (2.5% skim milk in wash buffer), were added to the wells. The plates were incubated for 90 minutes at RT. After five washes with the washing buffer, 50 μ l of OB1264 diluted with assay buffer was added to the wells, and the plates were incubated for 60 minutes at RT. Following three washes with washing buffer, 50 μ l of goat anti-Rabbit Immunoglobulin G (IgG)-HRP (1:1000, Bio-Rad) diluted in assay buffer was added to the wells, and the plates were incubated in the shade for 60 minutes at RT. After five washes with the washing buffer, 50 μ l of substrate solution (3,3',5,5'-Tetramethylbenzidine [TMB], Microwell Peroxidase Substrate System, KPL) was added to each well, and the plates were incubated in the shade for 30 minutes at RT. The reaction was stopped by adding 25 μ l of 2 N sulfuric acid, and the absorbance was measured at 450 nm using a Benchmark microplate reader (Bio-Rad).

Cell culture

293T cells (RIKEN BRC, Tsukuba, Japan) were cultured in DMEM supplemented with 10% Fetal Calf Serum (FCS) (BioWest, France), penicillin, and streptomycin.

Rat primary OPCs were prepared from the optic nerve using sequential immunopanning. The addition of Platelet-derived growth factor (PDGF) and Insulin-like growth factor (IGF) to the medium promotes cell survival (Barres et al., 1992, Lian et al., 2016). OLGs were induced from OPCs by depleting mitogens (Barres et al., 1992). Neurons were generated from a human neural precursor cell line (ReN cells, Merck) as previously described (Donato et al., 2007). In brief, the cells were seeded at a density of 30,000 cells/cm² in a 96-well plate and cultured until confluence. The medium was then replaced with one lacking growth factors, and differentiation was allowed to proceed for 4-7 days, followed by fixation for immunocytochemistry. For lower seeding densities, 1 mM dibutyryl-cAMP and 2 ng/ml Glial cell line-derived neurotrophic factor (GDNF) were added to the medium to promote differentiation.

Expression vectors

Mouse *Ecr4* fragments, 32-70 and 71-132, were amplified from pCMS-EGFP-mEcr4

(Kujuro et al., 2010) using KOD plus-Ver.2 polymerase according to the supplier's instructions (TOYOBO, Japan) and then inserted into pFUSE-hIgG1Fc2 (InvivoGen) to produce pFUSE-mEcrG4 (32-70)-hIgG1Fc2 (32-70) and pFUSE-mEcrG4 (71-132)-hIgG1Fc2 (71-132). Sense and anti-sense oligonucleotides for fragment 133-148 were annealed and inserted into pFUSE-hIgG1Fc2 to produce pFUSE-mEcrG4 (133-148)-hIgG1Fc2 (133-148). The following DNA primers were synthesized: for 32-70, the 5' primer was 5'-TGATATCGAACAACTCAAGAAGATGCTCC-3' and 3' primer was 5'-AAGATCTTCGTTTGGCAGCTTCAGG-3'; for 71-132, the 5' primer was 5'-TGATATCGCAGCTGTGGGACCGTACG-3' and 3' primer was 5'-AAGATCTGTGGGGACCAATGGCCGCA-3'; for 133-148, the 5' primer was 5'-ATCGAGCCGGGAAAGCTTCAGGCATGGAGCCAGTGTCAACTATAATGACTATA-3' and 3' primer was 5'-GATCTATAGTCATTATAGTTGACACTGGCTCCATGCCTGAAGCTTCCCGGCTCGAT-3'. The nucleotide sequences of the cloned complementary DNAs (cDNAs) were verified using the BigDye Terminator Kit version 3.1 (Thermo Fisher Scientific) and an ABI sequencer model 3130xl (Thermo Fisher Scientific).

In this study, *E. coli* DH5 α competent cells (Takara) were used as the host for plasmid amplification. The basic cloning procedure was as follows: First, the target gene fragment and the plasmid vector were double-digested using restriction endonucleases (NEB). The digested products were separated by agarose gel electrophoresis and purified using a gel recovery kit. Subsequently, the purified insert and linearized vector were mixed at an appropriate molar ratio and ligated using T4 DNA ligase (Takara) at 16°C overnight. The ligation mixture was then added to ice-thawed DH5 α competent cells, followed by incubation on ice, heat shock at 42°C for 45 seconds, and a further 2 minutes on ice. After adding LB liquid medium (BD) and recovering for 1 hour, the transformed bacterial suspension was evenly spread onto LB solid plates containing the appropriate antibiotic and incubated overnight at 37°C in an inverted position. The next day, single colonies were picked and validated using a colony PCR identification kit (TaKaRa). Positive clones were confirmed by DNA sequencing. Finally, plasmids were extracted using a plasmid miniprep kit (Omega Bio-tek) for subsequent experiments. The approval number for the plasmid construction and gene recombination experiments conducted in this study is 2022-060.

Western blotting (WB)

WB was performed as previously described (Takanaga et al., 2009). Briefly, 293T cells

were transfected with the vectors, pFUSE-hIgG1Fc2, 32-70, 71-132 and 133-148, using EndoFectin according to the supplier's instructions (GeneCopoeia). Three days after transfection, the cell extracts were prepared in the Radio-Immunoprecipitation Assay (RIPA) buffer, electrophoresed and transferred to a Hybond-P membrane (Amersham). The blotted membranes were probed for the following antigens: Ecrg4 (1 µg/ml, OB1264), Ecrg4 (1 µg/ml, 2A8) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, anti-mouse Ab, Chemicon). Mouse, Rabbit and Human IgG were detected using HRP-conjugated Goat anti-mouse IgG (1:5000, Santa Cruz Biotechnology), HRP-conjugated Goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnology) and HRP-conjugated Goat anti-Human IgG (1:5000, Bethyl Laboratories, TX), respectively. A chemiluminescence (ECL) system (Amersham) was used for detection and images were obtained using an ChemiDoc MP Imaging Lab system (Bio-Rad).

Immunocytochemistry

Immunostaining was performed as previously mentioned earlier (Kujuro et al., 2010, Nishide et al., 2009). 293T cells were transfected with the vectors as described above. Three days after transfection, the cells were fixed and immunostained with 2A8 (1 µg/ml) and OB1264 (1 µg/ml). Human, mouse and rabbit Fc were detected using Alexa 488-conjugated anti-human IgG (1:1000, ThermoFisher Scientific), Alexa 594-conjugated anti-mouse IgG (1:1000, ThermoFisher Scientific) and Alexa 594-conjugated anti-rabbit IgG (1:1000, ThermoFisher Scientific), respectively. Cells were counterstained with Hoechst 33342 (1 µg/ml, ThermoFisher Scientific) to visualize the nuclei.

Cell death was assessed using rabbit polyclonal anti-active Caspase 3 (1:1000, Cell Signaling Technology), followed by the Alexa 488-conjugated anti-rabbit IgG (1:1000, ThermoFisher Scientific). Cells were counterstained with Hoechst 33342 (1 µg/ml, ThermoFisher Scientific) to visualize the nuclei. Fluorescence images were captured using an AxioImager M1 microscope (Carl Zeiss).

Diagnosis of dementia, Alzheimer's disease and non-dementia patients

Patients were diagnosed according to the established guidelines for dementia, AD and MCI (Table 1) (Albert et al., 2011, Menck et al., 2017). The diagnosis of AD among dementia patients is based on the Consortium to Establish a Registry for Alzheimer's Disease Neuropsychological Assessment Battery (CERAD-NAB). Specifically, tests such as the Verbal Fluency Test, Boston Naming Test, Wordlist Encoding Test, Constructional Praxis Test,

Wordlist Recall Test, Wordlist Recognition Test, Constructional Praxis Recall Test, Intrusions Test, Wordlist Saving Test, Trail Making Test A, Trail Making Test B, and S-words Test are utilized to evaluate cognitive function (Fillenbaum and Mohs, 2023). The Mini Mental State Examination (MMSE) was partially used for dementia diagnosis (Folstein et al., 1975, Kaufer et al., 2008, Saxton et al., 2009). Non-dementia patients were diagnosed as indicated in Table 2.

Table 1. Classification of dementia and MCI patients

Patient age (years old)		-40	41-50	51-60	61-70	71-80	81-
Dementia	AD	M:1	M:1, F:1	M:10, F:4	M:10, F:13	M:23, F:35	M:9, F:23
	Normal Pressure Hydrocephalus			M:1	M:1, F:1	M:3, F:1	M:1, F:1
	Idiopathic Normal Pressure Hydrocephalus				M:2		
	Progressive nonfluent aphasia				F:1	F:1	
	progressive supranuclear palsy				F:1	F:1	
	Dementia with Lewy body				F:1	M:1	
	Frontotemporal lobar degeneration				M:1		
	Vascular Dementia					F:1	
	Neuronal intranuclear inclusion disease					F:1	
	others	F:1		M:1, F:1	M:1, F:1	M:3, F:4	
MCI				M:1	M:1, F:6	M:5, F:10	M:2, F:2

Patients were categorized by diagnosis, gender (M: male, F: female) and age

Table 2. Classification of non-dementia patients with neurological disorders

Patient age (years old)	-40	41-50	51-60	61-70	71-80	81-
Diagnosis	myospasm: M:1, myoclonus:F:1, migraine: F:1, syringomyelia: F:1, hysteria: M:1, limbic encephalitis: M:1, myopathy: M:1, Neuromyelitis optica: M:1, epilepsy: M:1, aseptic meningitis: M:1, F:1, psychogenic reaction: F:1, episodic ataxia: M:1, sarcoidosis: M:1, neuro-Behcet: F:1, tuberculous meningitis: M:1, femoral neuropathy: F:1, multiple cerebral infarction: F:1, multiple sclerosis:F:1, postresuscitation encephalopathy: F:1, myelopathy: M:1, fibromyalgia:F:1	dystonia: F:1, spastic paraplegia: M:1, multiple system atrophy: F:1, Huntington's disease: M:1, encephalitis: M:1, cervical dystonia: M:1, Cervical cord sarcoidosis: M:1, polymyositis:F:1, diabetic peripheral neuropathy: M:1, brachial neuritis: M:1, neuro-Behcet: F:1, immune-mediated neuropathy: M:1, syringomyelia:F:1, Cavernous vein sinus meninges: F:1, hypothalamic lesion: F:1	brachial plexus neuritis: M:1, F:1, thoracic myelopathy: M:1, leukoencephalopathy: F:1, aseptic meningitis: F:1, paraneoplastic neurological syndrome: M:1, cerebellar ataxia: M:1, peripheral neuropathy: F:1, muscular dystrophy:F:1, myopathy: M:1, spastic paraplegia: M:1, encephalopathy: F:1, cervical spondylotic myelopathy: F:1	hypertrophic pachymeningitis: M:1, multiple system atrophy: M:2, multiple cranial nerve palsy: M:1, myelitis: F:1, Muscle sarcoidosis: F:1, neurosarcoidosis: F:1, Sequelae of myelitis: F:2, limbic encephalitis: F:1, cerebellar ataxia: M:1, myelopathy: M:2, spastic paraplegia: M:1, alcoholic cerebellar atrophy: M:1, corticobasal degeneration: F:1, cerebral infarction sequelae: F:1, Left lower limb weakness: M:1, late-onset hypogonadism syndrome: M:1	spondylosis deformans: F:1, Pure akinesia: F:1, Sensory neuropathy: F:1, multiple system atrophy: M:1, myelitis: M:1, dural arteriovenous fistula: F:1, inclusion body myositis: M:1	spastic paraplegia: M:1, Multiple cranial nerve disorders: F:1, motor neuron disease: F:1, cranial polyneuritis: F:1

Human plasma and cerebrospinal fluid

Ethylenediaminetetraacetic acid (EDTA) -treated plasma and cerebrospinal fluid (CSF) samples were obtained from patients diagnosed with dementia, including AD and normal pressure hydrocephalus (NPH), as well as those with MCI and non-dementia neurological disorders, such as multiple system atrophy (MSA) and myopathy, using standard methods (Menck et al., 2017, Waybright et al., 2010). Plasma samples were also obtained from healthy eight individuals, as controls (Fig. 5). In brief, to prepare plasma, draw blood into EDTA-containing tubes, then centrifuge at 1,200 x g for 15 minutes at room temperature. Use

a valve filter to separate the plasma, transfer it into a 15 mL tube, and centrifuge again at 1,500 x g to remove cellular debris, yielding the final plasma sample. To prepare CSF samples, collect ventricular CSF in sterile tubes on ice, centrifuge at 2000 g for 10 minutes at 4 °C to clarify, and aliquot into vials for storage at -80 °C. After rapid freezing in liquid nitrogen, use 3 mL of CSF for cell counts, glucose, protein levels, and microbiological analysis. For proteomics, digest samples with trypsin, desalt using C-18 columns, and lyophilize for strong cation exchange (SCX) fractionation. Protein concentration is determined by Bicinchoninic Acid (BCA) assay, and aliquots are prepared for further analysis. Informed consent was obtained from all subjects for research purposes, and the study was approved by the ethical committee of Hokkaido University and Tokyo Metropolitan Institute for Geriatrics and Gerontology. The approval numbers for the clinical studies are 12-0001(6), and 19-0002(1).

A β 40 and A β 42 ELISA assay

A β 40 and A β 42 concentrations in plasma and CSF were measured using Human/Rat β Amyloid 40 and β Amyloid 42 ELISA Kits WAKO, following to the supplier's instructions (FUJI FILM). The representative standard curves for A β 40 and A β 42 are shown in Figure 6.

RNA sequencing analysis of the plasma of dementia, MCI and non-dementia patients

Total RNA containing miRNA was extracted from the patients' plasma using the miRNeasy kit for plasma/serum (QIAGEN) following the manufacturer's instructions and was subjected to RNA-seq analysis (Eurofins). The RNA-seq data has been deposited in GEO.

RT-qPCR

Total RNA from culture supernatants (culture sup) and cells was prepared using the miRNeasy for plasma/serum (QIAGEN) and the mirVana miRNA isolation kit (ThermoFisher), following the manufacturers' instructions. The total RNAs extracted from plasma and culture sup were converted to cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN), while total RNAs from the cells were converted using the ReverTra Ace qPCR RT Kit (TOYOBO). Additionally, miRCURY LNA serum/plasma samples (QIAGEN) were utilized to generate cDNA from small RNA including miRNA and piRNA.

Real-time PCR was conducted using the StepOnePlus system (Thermo Fisher Scientific) with the miRCURY LNA miRNA PCR assay (QIAGEN) and THUNDERBIRD® SYBR

qPCR Mix (TOYOBO) for let-7b (YP00204750) and mRNA, respectively.

The PCR conditions for mRNAs were as follows: 1 min at 95°C; followed by 40 cycles of 95°C for 15 sec, and 60°C for 30 sec. For let7b, the PCR conditions were as follows: 2 min at 95°C; followed by 40 cycles of 95°C for 10 sec, and 56°C for 1 min. All RNA expression data were normalized to the let7b (for plasma and culture sup) or 18S (for cultured cells) using the $\Delta\Delta C_t$ method. The following DNA primers for target mRNA were synthesized: for human SLC46A3,

the forward primer was 5' -ACCACATTTTGGGGAGCTTG-3' ,

the reverse primer was 5' -AGCCAGATGACAGTCCTGTT-3' . For human CHST3, the forward primer was 5' -GCAGGGCAACATCTTCTACC-3' ,

the reverse primer was 5' -CACAGGAAGAGCTGCTTGAG-3' . For rat Chst3, the forward primer was 5' -cagaaggcccagagatgta-3' , the reverse primer was

5' -aactgctccgaggagttctt-3' . For human TTC39A, the forward primer was

5' -CACACTTTTCGAATCCAGGCA-3' , the reverse primer was

5' -TCCAGCTGTCTCTGTCTTCC-3' . For 18S, the forward primer was

5' -CGGACAGGATTGACAGATTG-3' , the reverse primer was

5' -CAAATCGCTCCACCACTAA-3' .

MTT

Cell proliferation was measured using the MTT assay. Two thousand cells were seeded in each well of a 96-well plate with 100 μ L of culture medium. To assess cell proliferation, an MTT assay was performed by adding 5 μ L of MTT solution (5 mg/mL, 341-01823, Dojindo) on 3 day of culture, followed by 3 hours of incubation. The medium was then replaced with 100 μ L of Dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. Proliferation was measured using a microplate reader (Bio-Rad) at an absorption of 570 nm (Hide et al., 2009).

Animals

All used mice were 2 to 3 months old C57BL/6 mice. All mice experiments were performed following the protocols approved by the Animal Care and Use Committee of Hokkaido University. The approval numbers for the animal experiments is 21-0066.

Preparation of brain and plasma from mice injected with ECRG4 (71-107) into brain parenchyma

ECRG4 (71-107) was synthesized by GL Biochem (Shanghai) Ltd. Five microliters of

either ECRG4 (71-107) (20 μ M in 1% DMSO-PBS) or 1% DMSO-PBS alone (DMSO) was injected into the brain parenchyma of 8 to 10 week-old C57BL/6 mice every other days, for a total of five injections. The stereotactic injection coordinates were 2 mm anterior from the lambda, 2 mm lateral from the sagittal suture, and 3 mm deep. Two days after the last injection, the brains and blood of the mice were collected. The brains were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) and kept at -80°C until use, while plasma was prepared using a standard method. Sagittal sections (12 μ m thick) of the brains were prepared and immunolabeled with anti-GalC for OLGs, anti-CD31 for endothelial cells, anti-Platelet-derived growth factor receptor alpha (PDGFR α) for OPCs and anti-IgG for blood.

Preparation of human brain

Hippocampal sections were prepared from the brains of non-AD, EAD, and AD patients as previously described (Kizuka et al., 2015). Informed consent was obtained from all subjects for research purposes, and the study was approved by the ethical committees of Hokkaido University and Tokyo Metropolitan Institute for Geriatrics and Gerontology. For human brain analysis, the hippocampal region of the human brain was embedded in OCT and sectioned into 8 μ m frozen slices. The sections were fixed in 4% PFA for 10 minutes and then washed three times with PBS. The sections were treated with HistoVT solution at 70°C for 20 minutes. Blocking was performed at room temperature for two hours using a blocking buffer containing 3% skim milk and 0.3% Triton X-100. The sections were washed three times and incubated with the primary Ab overnight at room temperature. After three times washes, the sections were incubated with the secondary Ab at room temperature for one hour, followed by three times washes. The sections were then blocked with 1% Sudan Black solution for five minutes and washed with 70% ethanol. Finally, the sections were mounted using mounting medium (Dako, S3023). The sections were immunolabeled with Ulex europaeus agglutinin 1 (UEA1) for endothelial cells, Neural/glial antigen 2 (NG2) for OPCs and GFAP for astrocytes. Fluorescence images were captured using an AxioImager M1 microscope (Carl Zeiss) and subsequently quantified using ImageJ software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Data were analyzed using an unpaired two-tailed t-test for between-group comparisons or a one-way analysis of variance (ANOVA) with Tukey's post-hoc test as indicated in the figure legends. All results

are expressed as the mean $\pm$ standard deviation (SD) values. All experiments were conducted more than three times with similar results.

Immunization of mice

ECRG4, mixed with adjuvant (TiterMax® Gold, CYT, G-1) as antigen, were injected BALB/c mouse's single tail base every two weeks (Fig. S1). Blood was collected weekly to verify the Ab concentration in the serum.

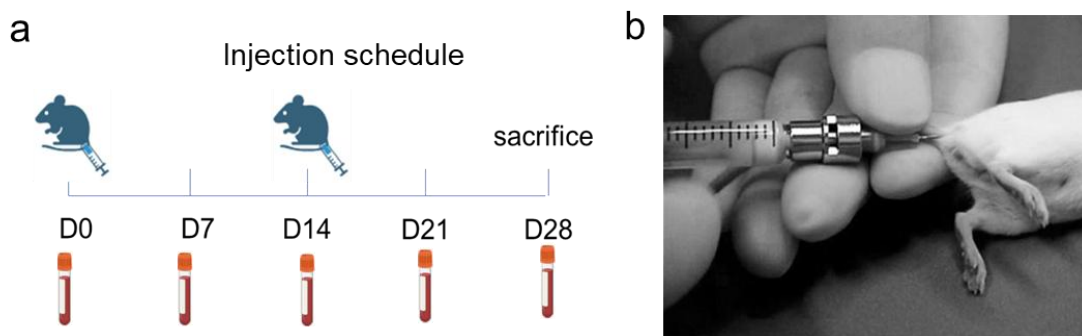


Fig. S1. Mouse immunization process.

(a) Time course of mouse immunization. Antigen were injected into the single tail base of mice on D0 and D14. Blood samples were collected once a week to detect Ab concentrations in mice. Mice were sacrificed on D28 and B cells from their spleens and lymph nodes were collected.(b) Schematic diagram of the injection method (Sado et al., 2006). The injection method at the single tail base position is based on the article by Sado et al.

Cell fusion and screening

Lymph node and spleen were extracted from mouse and cell suspension was prepared by 70 μ m filter. The prepared allogeneic myeloma cells were mixed with mouse lymphocytes in proportion and the fusion promoter polyethylene glycol solution (PEG, sigma, P7181-5X5ML) was added. Under the action of PEG, various B cells can fuse with myeloma cells to form hybridoma cells. And then, the fusion cells were screened by selecting HAT medium. Hybridoma cells were cloned and cultured by limited dilution method. After several rounds of screening, stable Ab-producing hybridoma cells were obtained. I collected the culture medium of these hybridoma cells and collected the mAbs in the culture medium through ProG beads (Cytiva, 17061801).

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

First, the Ab-bound ProG beads and supernatant were separately mixed with SDS sample buffer and heated at 95°C for 5 minutes to denature and linearize the proteins. Then, the stacking gel and separating gel were prepared, with the separating gel poured into the gel plate and allowed to solidify before the stacking gel was added. Next, the samples were loaded into the wells of the stacking gel and placed in the electrophoresis chamber. Under the influence of an electric field, the SDS-coated proteins migrated based on their molecular weight. After electrophoresis, the gel was typically stained with Coomassie Brilliant Blue and then destained to reveal protein bands. Finally, the molecular weights of the proteins were analyzed by comparing them with standard molecular weight markers.

6. Results

Development of an ELISA for ECRG4 detection

For a purpose of establishing a new ECRG4 sandwich ELISA system, I generated four monoclonal Abs and one polyclonal Ab against different regions of ECRG4 (Fig. 1).

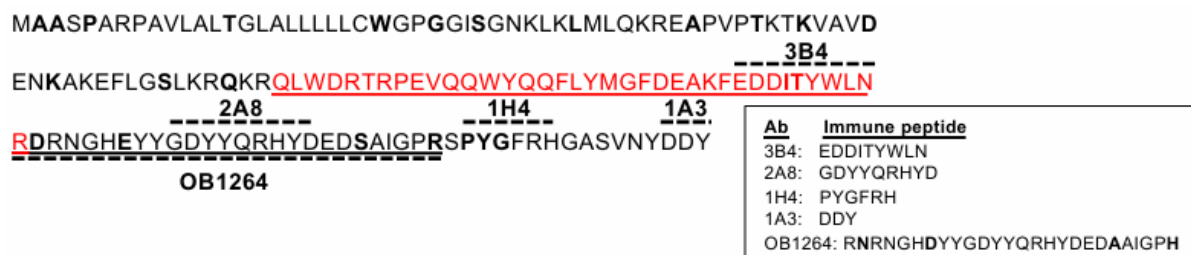


Fig. 1. Amino acid sequence of ECRG4 and the recognition sites of various ECRG4 Abs.

Information on immunized peptides (dashed line) related to the human ECRG4 amino acid (AA) sequence. OB1264 was generated using the mouse EcrG4 peptide. The differences between mouse and human ECRG4 AA sequences are indicated in bold. Mouse and rabbit Abs are shown top at the top and bottom of the AA sequence, respectively. The solid underline indicates the region of ECRG4 (71-132). ECRG4 (71-107) is shown in red.

I then examined which combination of mouse and rabbit Abs is the best to detect ECRG4. As shown in Fig. 2A, I found that the signal intensity for mAbs, 3B4 and 1A3, remained almost unchanged, whereas the signals for mAbs, 1H4 and 2A8, increased along with increase of concentration of the applied ECRG4 peptide. In particular, 2A8 demonstrated a broader linear dynamic range in compared to 1H4 (Fig. 2a). Therefore, I decided to use 2A8 Ab as capture Ab for following ELISA development.

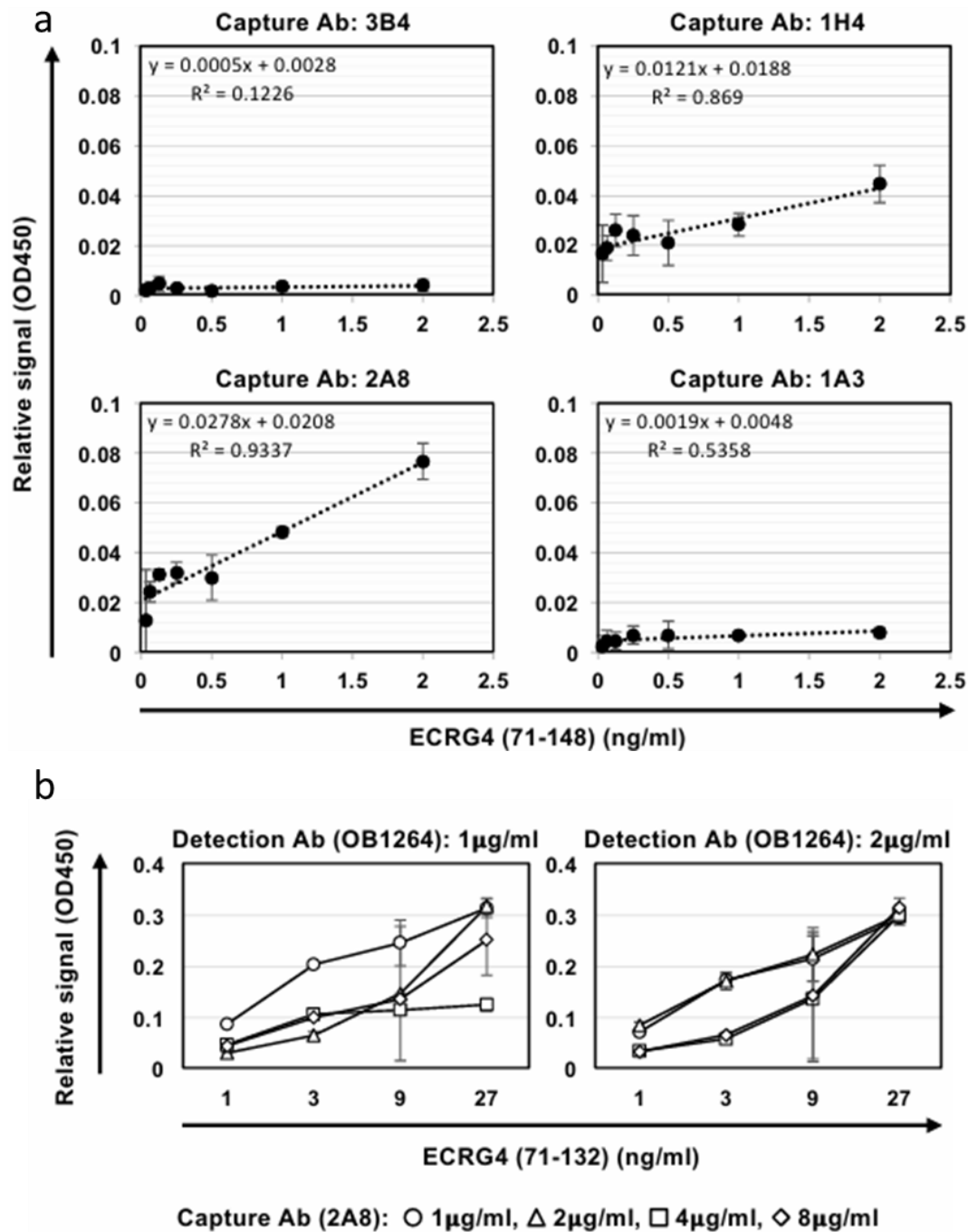


Fig. 2. Development of ELISA system for ECRG4 detection.

(a) ECRG4 (71-148) peptide spread onto multi-well plates at several concentrations were incubated with indicated ECRG4 Abs. Amount of ECRG4 Abs were detected by OB1264 and measured with a HRP-conjugated indicated rabbit Ab.

(b) 2A8 Ab spread onto multi-well plates at several concentrations were incubated with ECRG4 (71-132) at several concentrations. Several concentration of OB1264 Ab were added and were incubated with HRP-conjugated indicated rabbit Ab. Amount of ECRG4-bound Abs were measured by incubation with a HRP substrate.

To determine the optimal concentrations of the capture Ab and detection Ab, I evaluated the sensitivity of the ELISA system using various concentrations of 2A8 as the capture Ab and OB1264 as the detection Ab. Regardless of the combination of concentrations, the signal increased with increasing concentrations of ECRG4. The system exhibited the broadest linear range at low concentrations of ECRG4 particularly when both 2A8 and OB1264 were used at a concentration of 1 $\mu\text{g/ml}$. Thus, I selected this combination of the Abs and concentration for ECRG4 detection (Fig. 2b).

To further characterize the specificity of 2A8 and OB1264, I overexpressed different regions of ECRG4 in 293T cells, and then, analyzed by WB and immunohistochemistry with the Abs. In WB, both Abs specifically exhibited bands at positions corresponding to the amino acid 71th to 132nd of ECRG4 protein (ECRG4 (71-132)) fragment (Fig. 3a). Similarly, in immunostaining, these two Abs specifically bound to the cells expressing ECRG4 (71-132) (Fig. 3b, c), indicating that 2A8 and OB1264 Abs specifically recognize the ECRG4 (71-132) fragment.

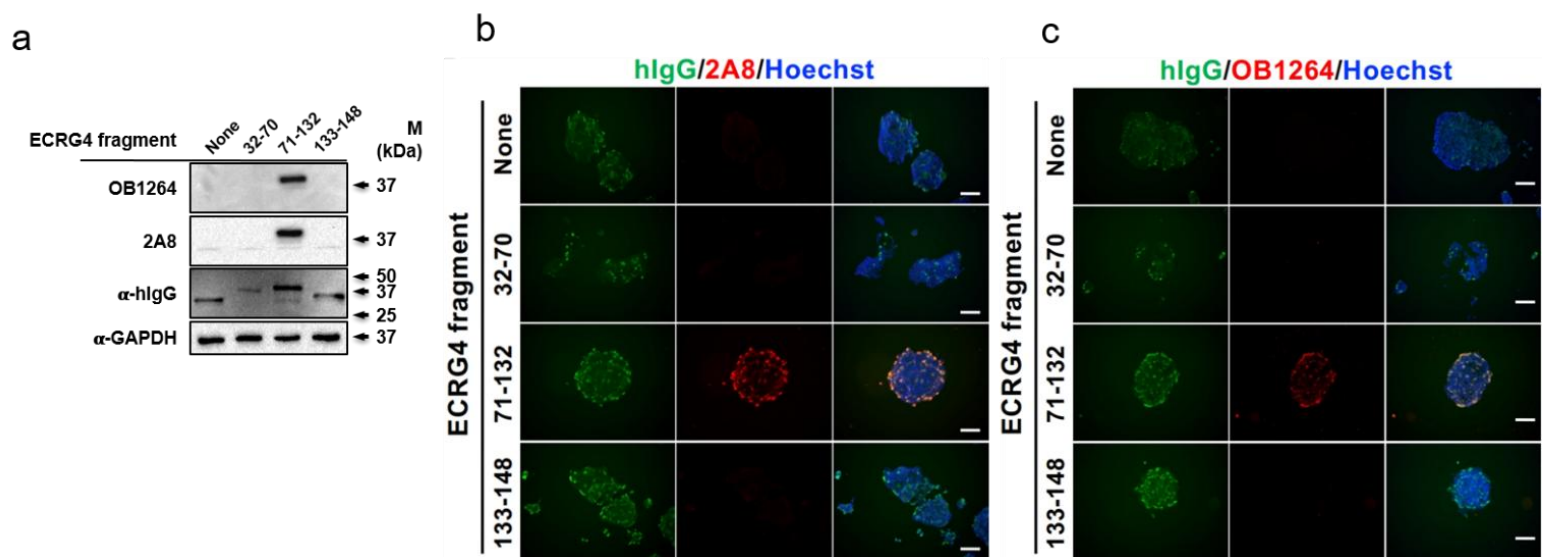


Fig. 3. Characterization anti-ECRG4 Abs.

(a) Various ECRG4 fragments fused with human IgG (hIgG) were expressed in 293T cells, expressed ECRG4-hIgG were collected by co-immunoprecipitation, and then applied for WB using anti-hIgG (α -hIgG), OB1264, and 2A8. GAPDH was detected as internal control.

(b, c) Representative immunostaining pictures of expressed various ECRG4 fragments fused with human hIgG by anti-hIgG (hIgG) and 2A8 (b) or OB1264 (c). Scale bar: 100 μm .

According to overall results, I decided to use 2A8 as capture Ab and OB1264 as detection Ab, respectively, in the ELISA system for ECRG4 detection in fluid samples of human subjects.

Increase of ECRG4 in plasma of patients with dementia and MCI

To investigate whether ECRG4 could serve as a potential biomarker for the diagnosis of AD, I measured ECRG4 level in the plasma collected from non-dementia but with neurological disorder subjects (non-dementia), subjects showing mild cognitive impairment (MCI), dementia patients, and AD patients by the established ELISA system (Table 1, 2). ECRG4 level in the plasma of AD patients are higher than those of non-dementia subjects (Fig. 4a). Level of ECRG4 in plasma remains high after including the samples of MCI and non-AD dementia patients in the sample of AD patients (dementia + MCI) (Fig. 4a). Increase of ECRG4 in plasma was detected regardless of gender (Fig. 4b).

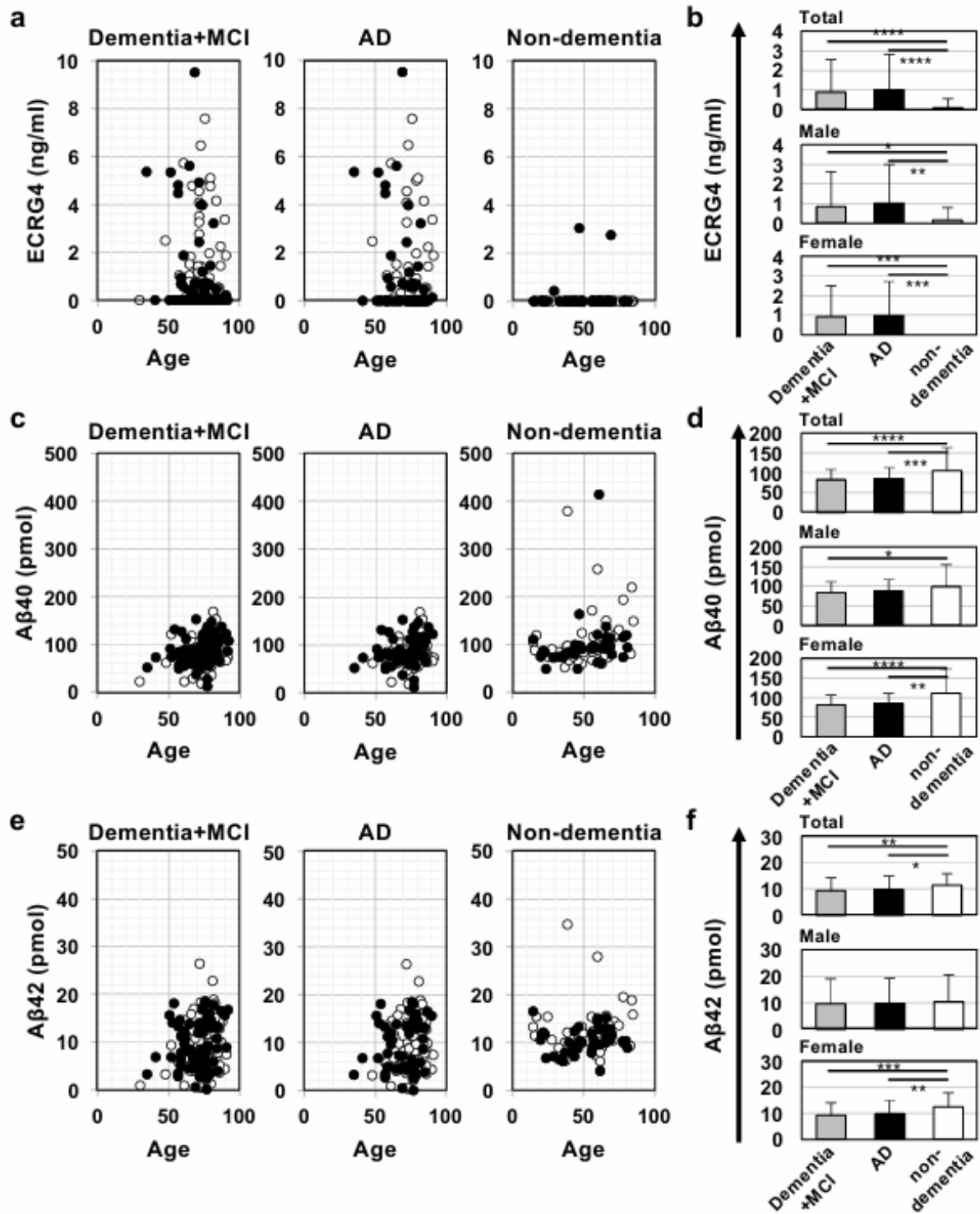


Fig. 4. Increase of ECRG4 in plasma of patients with dementia or mild cognitive impairment.

(a, b) Level of ECRG4 in the plasma collected from non-demented subjects, Alzheimer disease patients (AD), and subjects presenting dementia or mild cognitive impairment (MCI) were measured by ELISA utilizing anti-ECRG4 Abs. The values were plotted against age of subjects (a) and separately in each sex and in total (b).

(c, d) Level of A β 40 in the plasma collected from non-demented subjects, AD, and subjects presenting dementia or MCI was measured by ELISA. The values were plotted against age of subjects (c) and separately in each sex and in total (d).

(e, f) Level of A β 42 in the plasma collected from non-demented subjects, AD, and subjects presenting dementia or MCI was measured by ELISA. The values were plotted against age of subjects (e) and separately in each sex and in total (f).

The number of people in each group was 159 (dementia + MCI), 129 (AD), and 80 (non-dementia). Empty circle, males; filled circle, females. Error bars represent mean $\pm$ SEM.

*P<0.05, **P<0.01, ***P<0.005, ****P<0.001 (Tukey's multiple comparison tests).

ECRG4 was detected in 47.3%, 60%, and 25% of AD, non-AD, and MCI patients in their plasma, respectively, while ECRG4 was detected in only 5% of non-dementia patients in their plasma (Table 3). Pattern of the population is similar between each gender group except MCI and non-dementia patients (Table 3). ECRG4 was approximately detected in 46% and 60% of AD and non-AD dementia patients, respectively, in both male and female group, while ECRG4 was detected in 9.1% and 35.3% of male and female MCI patients, respectively, and in 10% and 0% of male and female non-dementia patients, respectively (Table 3). In terms of the relationship between level of ECRG4 in plasma and the score of MMSE, which is a test for evaluation of cognitive function of the subjects (Arevalo-Rodriguez et al., 2015), more than 30% of patients with cognitive impairment (MMSE $\leq$ 23) was ECRG4 positive in plasma (34.3% in total, 32.1% in male, 38.9% in female subjects), while 12.9% (16.7% in male, 10.5% in female subjects) and 22.2% (10% in male, 37.5% in female subjects) was positive in the patients scoring MMSE 24–27 and MMSE $\geq$ 28, respectively (Table 4).

Table 3. Percent of ECRG4-positive patients with dementia, MCI and non-dementia with neurological disorders

Diagnosis		total	male	female
Dementia	AD	47.3% (61/129)	44.4% (24/54)	49.3% (37/75)
	non-AD	60% (18/30)	64.3% (9/14)	56.3%(9/16)
Mild cognitive impairment		25% (7/28)	9.1% (1/11)	35.3% (6/17)
Non-dementia		5% (4/80)	10% (4/40)	0% (0/40)

Table 4. Mini Mental State Examination (MMSE) distribution of the dementia and MCI patients with increased plasma-ECRG4

Cognitive functioning tests	total	male	female
MMSE ≤ 23	34.3% (23/67)	32.1% (11/21)	38.9% (12/46)
MMSE 24~27	12.9% (4/31)	16.7% (2/12)	10.5% (2/19)
MMSE ≥ 28	22.2% (4/18)	10.0% (1/10)	37.5% (3/8)

On the other hand, ECRG4 was not detected in plasma of healthy subjects (Fig. 5a), suggesting that ECRG4 increases in plasma in dementia patients including AD patients.

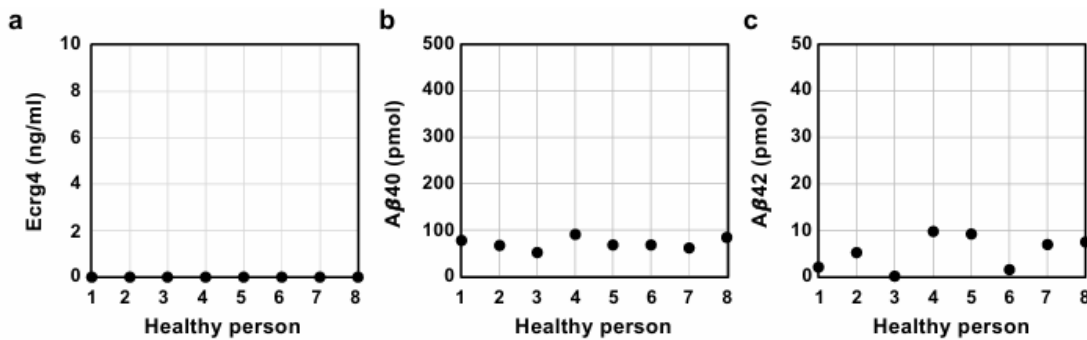


Fig. 5. Concentrations of ECRG4 and Aβs in plasma of the healthy subjects.

Level of ECRG4 (a), Aβ40 (b), and Aβ42 (c) in plasma samples collected from healthy subjects were measured by ELISA (N = 8).

Subsequently, I measured levels of Aβ40 and Aβ42 in patient plasma, which is used as biomarkers for AD (Brand et al., 2022, Nakamura et al., 2018). Initially, I plotted a standard curve using known concentrations of Aβ commercially available ELISA kits (Fig. 6a, b). Then, by using these ELISA kits, Aβ concentrations in plasma samples of subjects were measured. Levels of Aβ40 and Aβ42 were lower in plasma samples of AD patients, which are consistent with the data of previous articles (Nakamura et al., 2018, Schindler et al., 2019) (Fig. 4c–f). Level of Aβ40 and Aβ42 in plasma remains low after including the samples of MCI and non-AD dementia patients in the sample of AD patients (dementia + MCI) (Fig. 4c–f). Low level of Aβ42 was detected in both dementia + MCI group and AD patients in female subjects, while low level of Aβ40 was detected only in dementia + MCI group in male subjects (Fig. 4d, f).

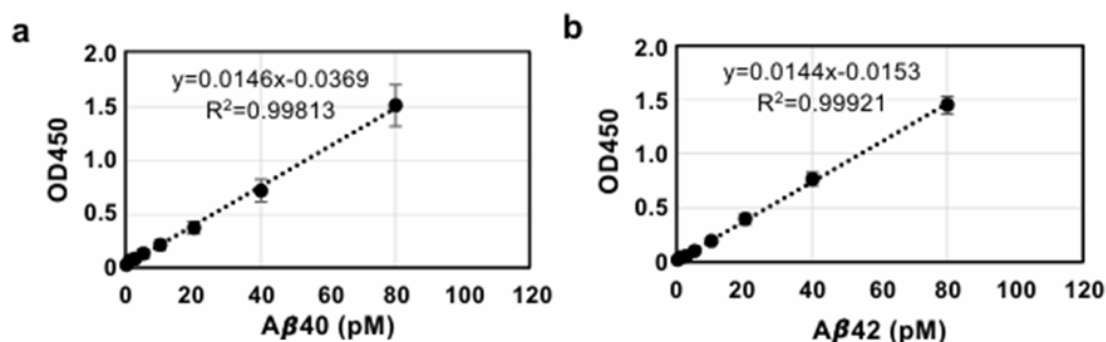


Fig. 6. Measurement of amyloid β-proteins by ELISA.

By using ELISA kits, Human/Rat β Amyloid 40 ELISA kit (WAKO) for Aβ40 (a) and Human/Rat β Amyloid 42 ELISA kit (WAKO) for Aβ42 (b), synthetic Aβ was applied on the plate and the bound Aβ was detected with the provided detection Abs conjugated with HRP according to the provided protocol.

On the other hand, the ratio of Aβ42/Aβ40 was not different among dementia + MCI group, AD patients, and non-dementia patients (Fig. 7a, b), suggesting that change of ECRG4 level in plasma can sensitively reflect the onset of cognitive impairment and dementia of patients.

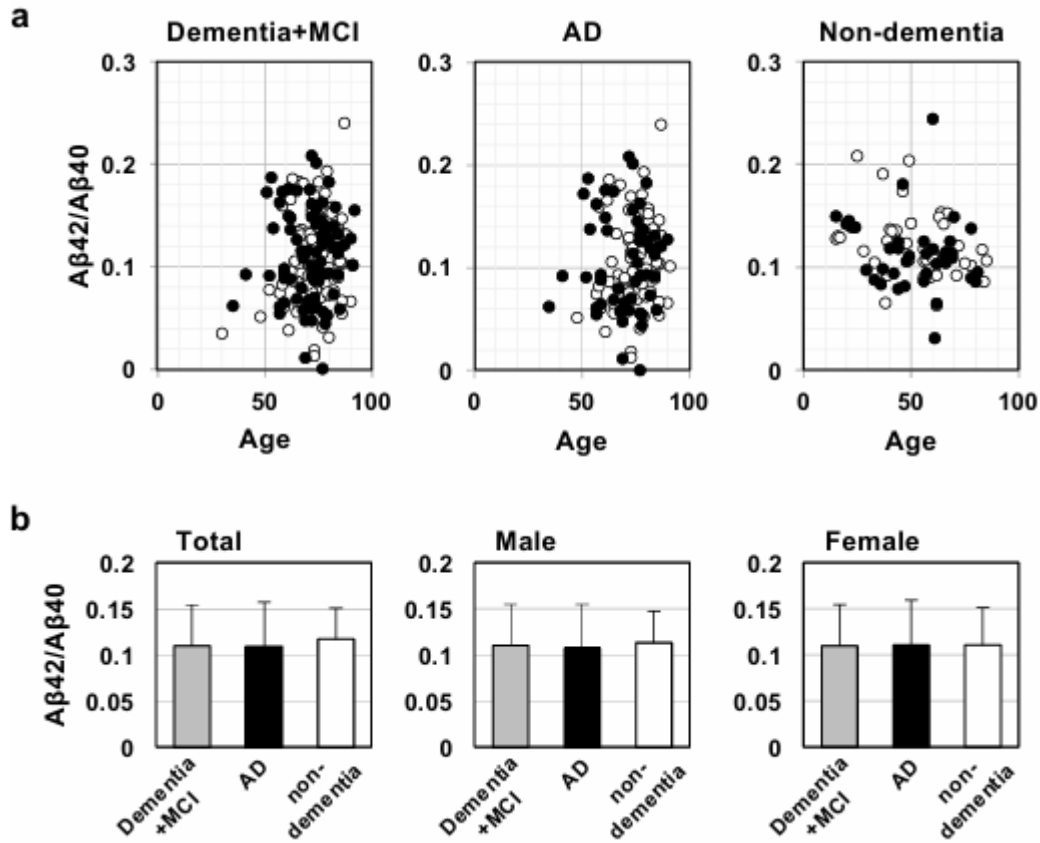


Fig. 7. Aβ42/Aβ40 ratio in plasma of the examined subject.

(a, b) Ratio of the level of Aβ42 and Aβ40 (Aβ42/Aβ40) in plasma of the examined subjects were calculated based on data of Fig. 4c and e. AD: Alzheimer disease patients, MCI: mild cognitive impairment patients. The values were plotted against age of subjects (a) and separately in each sex and in total (b). The number of people in each group was 159 (dementia + MCI), 129 (AD), and 80 (non-dementia). Empty circle, males; filled circle, females. Error bars represent mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$ (Tukey's multiple comparison tests).

I also examined CSF samples, which is another body fluid that is frequently used for AD biomarker examination (Mori et al., 1995). There was no difference in ECRG4 levels between AD, dementia + MCI, and non-dementia groups regardless of gender of subjects (Fig. 8a, b). Levels of Aβ40 and Aβ42 were significantly lower in both dementia + MCI group and AD patients regardless of gender of subjects, which is consistent with other studies data (Aschenbrenner et al., 2022, Hansson et al., 2007, Shoji and Kanai, 2001) (Fig. 8c–f). In terms of Aβ42/Aβ40 ratio, the value was lower in AD patients and in dementia + MCI group than that of non-dementia patients in total population, while the value is lower only in dementia + MCI group in female subjects (Fig. 8g, h).

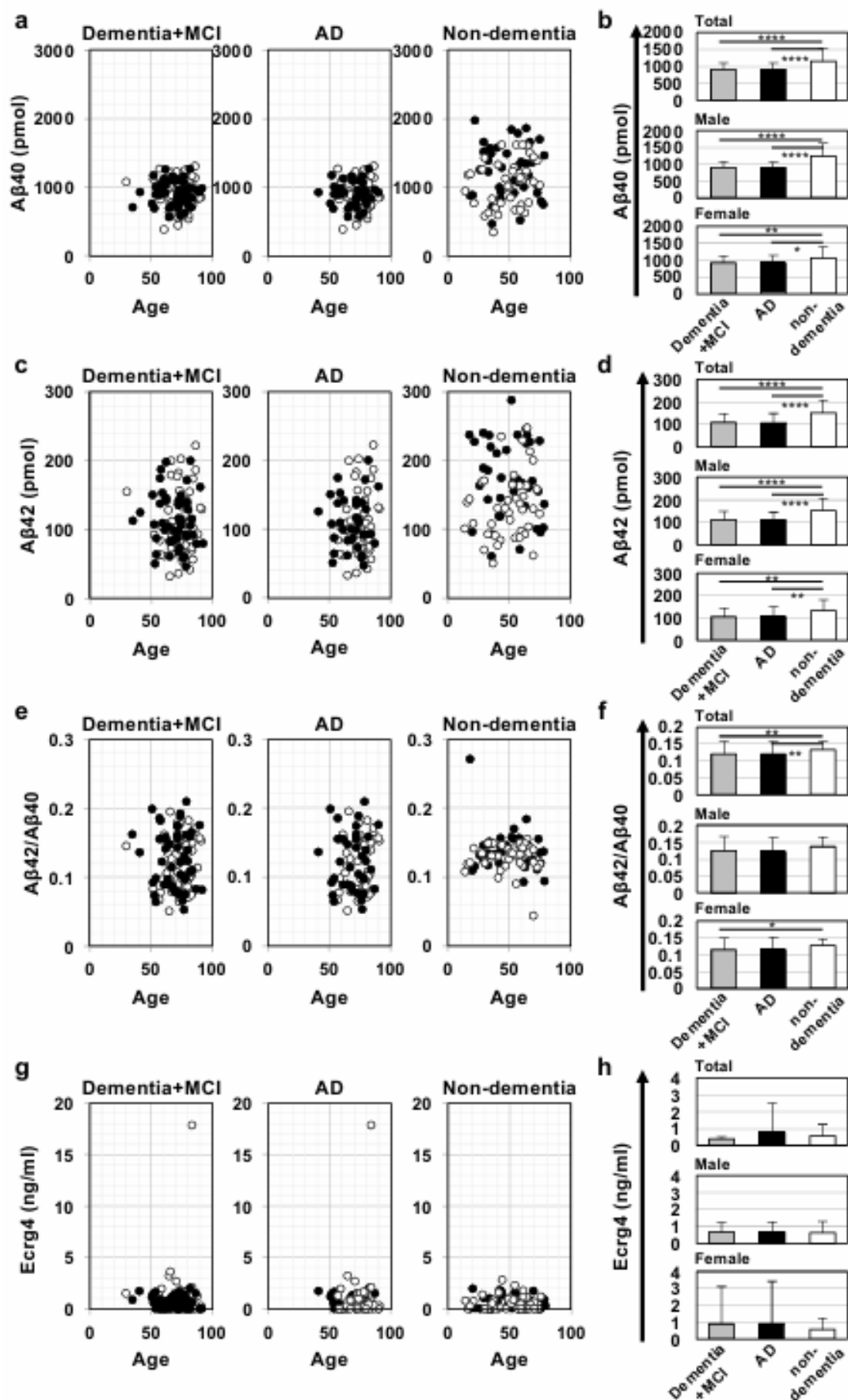


Fig. 8. Levels of ECRG4 and amyloid β -proteins in cerebrospinal fluid of the examined

subjects.

(a, b) Level of ECRG4 in the CSF collected from non-demented subjects, Alzheimer disease patients (AD), and subjects presenting dementia or MCI was measured by ELISA utilizing anti-ECRG4 Abs. The values were plotted against age of subjects (a) and separately in each sex and in total (b).

(c, d) Level of A β 40 in the CSF collected from non-demented subjects, AD, and subjects presenting dementia or MCI was measured by ELISA. The values were plotted against age of subjects (c) and separately in each sex and in total (d).

(e, f) Level of A β 42 in the CSF collected from non-demented subjects, AD, and subjects presenting dementia or MCI was measured by ELISA. The values were plotted against age of subjects (e) and separately in each sex and in total (f).

(g, h) Levels of A β 42/A β 40 ratio were calculated based on data of (c) and (e). The values were plotted against age of subjects (g) and separately in each sex and in total (h). The number of people in each group was 159 (dementia + MCI), 129 (AD), and 80 (non-dementia). Empty circle, males; filled circle, females. Error bars represent mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$ (Tukey's multiple comparison tests).

Spearman correlation analyses revealed that plasma ECRG4 levels were significantly negatively correlated with A β 40 ($\rho = -0.437$, $p < 0.001$), A β 42 ($\rho = -0.455$, $p < 0.001$), and the A β 42/A β 40 ratio ($\rho = -0.305$, $p < 0.001$) (Fig. 9a-c). To evaluate the predictive value of plasma ECRG4, multivariate logistic regression analysis was performed. ECRG4 ($p = 0.001$, OR = 2.253), A β 40 ($p = 0.001$, OR = 0.925), A β 42 ($p = 0.001$, OR = 2.040), A β 42/A β 40 ratio ($p = 0.007$, OR < 0.001), and age ($p < 0.001$, OR = 1.091) were identified as independent predictors of AD (Fig. 9d-e).

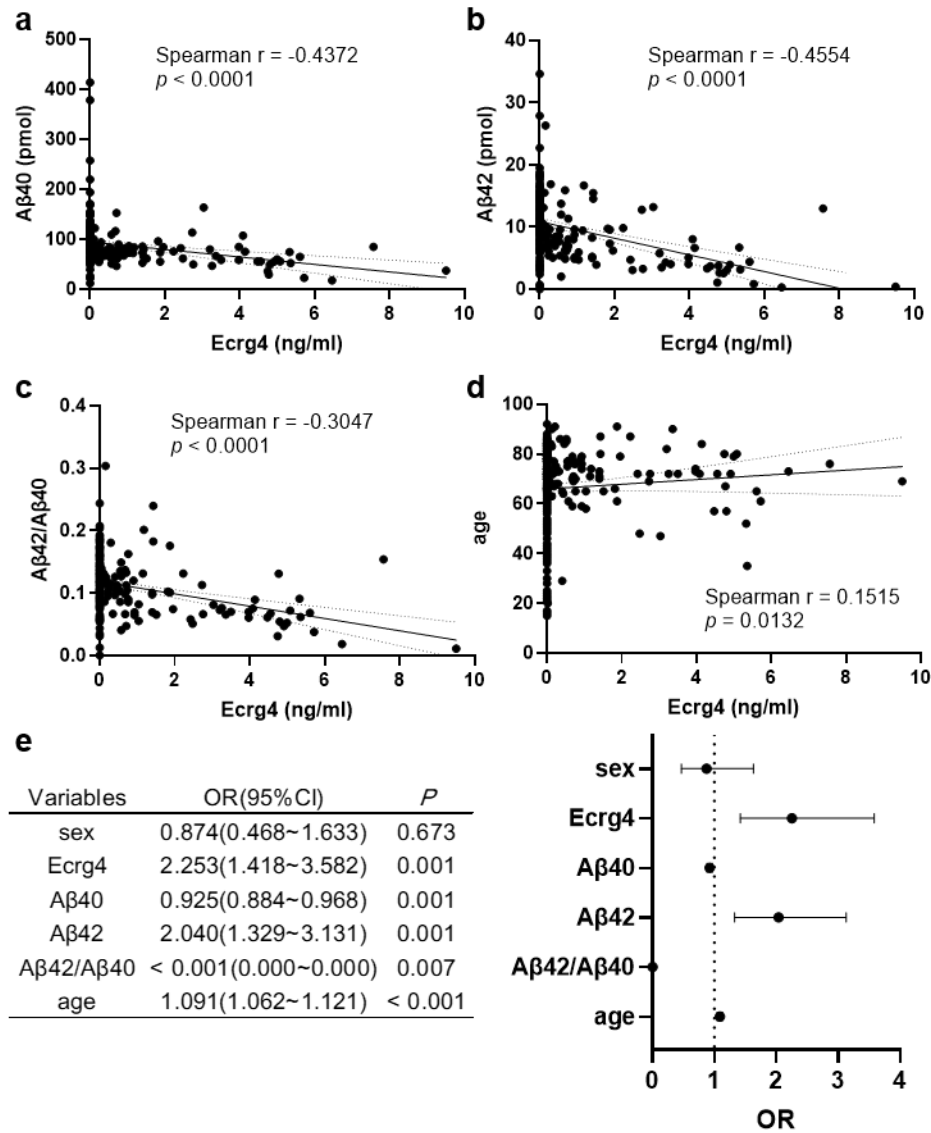


Fig. 9. Association between plasma ECRG4 levels and AD biomarkers and disease status.

Scatter plots illustrating the Spearman correlation of plasma ECRG4 levels with (a) A β 40, (b) A β 42, (c) A β 42/A β 40 ratio, and (d) age (years). Each panel displays Spearman's r and the corresponding p -values. (e) Forest plot presenting the odds ratios (ORs) and 95% confidence intervals (CIs) for each variable included in the multivariate logistic regression model, comparing the MCI and AD groups against the non-dementia and non-AD control group. The model was adjusted for all variables.

Overall, these results indicate that ECRG4 increases in plasma of dementia patients including AD patients. Additionally, ECRG4 in plasma can be a novel biomarker of dementia patients.

ECRG4 induces cell death in OLGs

To understand the mechanism underlying the increase of ECRG4 in plasma of dementia and MCI patients, I performed RNA-seq analysis on plasma samples of ECRG4-positive and -negative subjects in dementia + MCI group. Several RNA species including miRNA, piRNA, tiRNA, and mRNA were detected in the plasma samples (Fig. 10a-d).

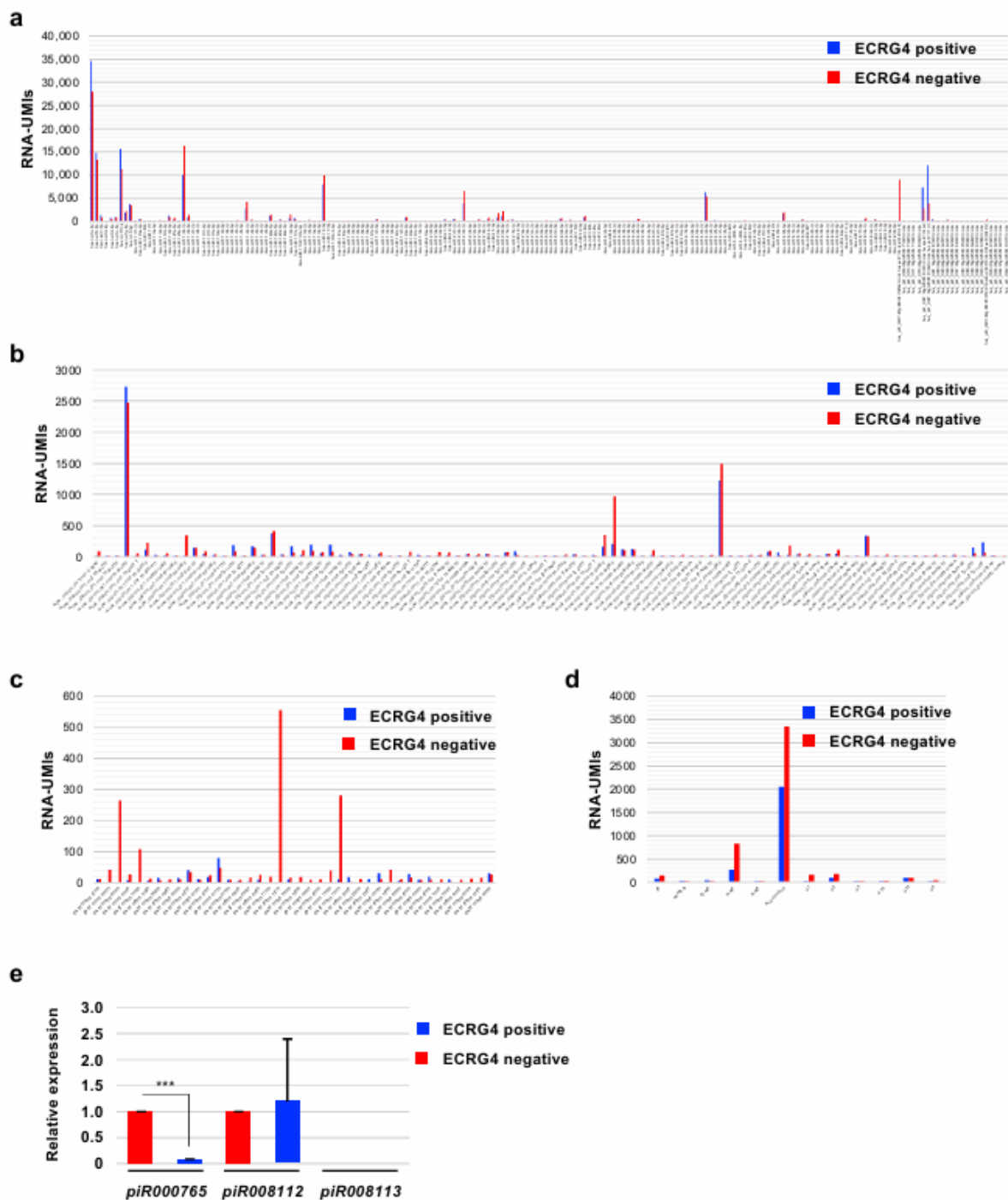


Fig. 10. A panel of detected RNA species in RNA sequencing analysis of plasma of the

subjects presenting dementia or mild cognitive impairment.

(a-d) A panel of RNAs detected in the plasma of subjects presenting dementia or MCI. The plasma samples were divided into the ECRG4-positive (empty columns) and -negative (filled columns) groups, were combined in each group (N = 30 in EcrG4-positive group, N = 63 in EcrG4-negative group), and then, RNA expressions were examined by RNA sequencing. RNA-UMIs, RNA-Unique Molecular Identifiers (UMIs). miRNA and piRNA (a); tiRNA (b); mRNA (c); other RNA (d). (e) Top three piRNAs detected in RNA sequencing analysis (a) were further analyzed by quantitative PCR (q-PCR). Blue and red columns represent the data of ECRG4-positive and -negative samples.

Among them, top three piRNAs (*piR000765*, *piR008112* and *piR008113*) and mRNAs (*solute carrier family 46 member 3 (SLC46A3)*, *carbohydrate sulfotransferase (CHST3)*, and *tetratricopeptide repeat domain 39A (TTC39A)*) showing significant difference of expression level between ECRG4-positive and -negative samples were selected for further validation by qPCR analysis (Fig. 11a, Fig. 10a). Expression level of *piR000765* was significantly reduced among the examined piRNAs (Fig. 10e), whereas the expression levels of all three mRNAs (*CHST3*, *SLC46A3*, and *TTC39C*) were reduced (Fig. 11b). Currently, the functional role of *piR000765* remains undefined based on the available literatures. On the other hand, it is known that *SLC46A3* and *TTC39C* are mainly associated with neurons, while *CHST3* is mainly associated with OLG lineage cells (the human protein atlas: <https://www.proteinatlas.org/>), suggesting that the increase of ECRG4 in plasma originates in alterations of the brain-resident cells, and, further, that an impairment of BBB function could contribute to the ECRG4 increase in plasma via a leakage of ECRG4 from brain since ECRG4 is mainly expressed in brain (Gonzalez et al., 2011).

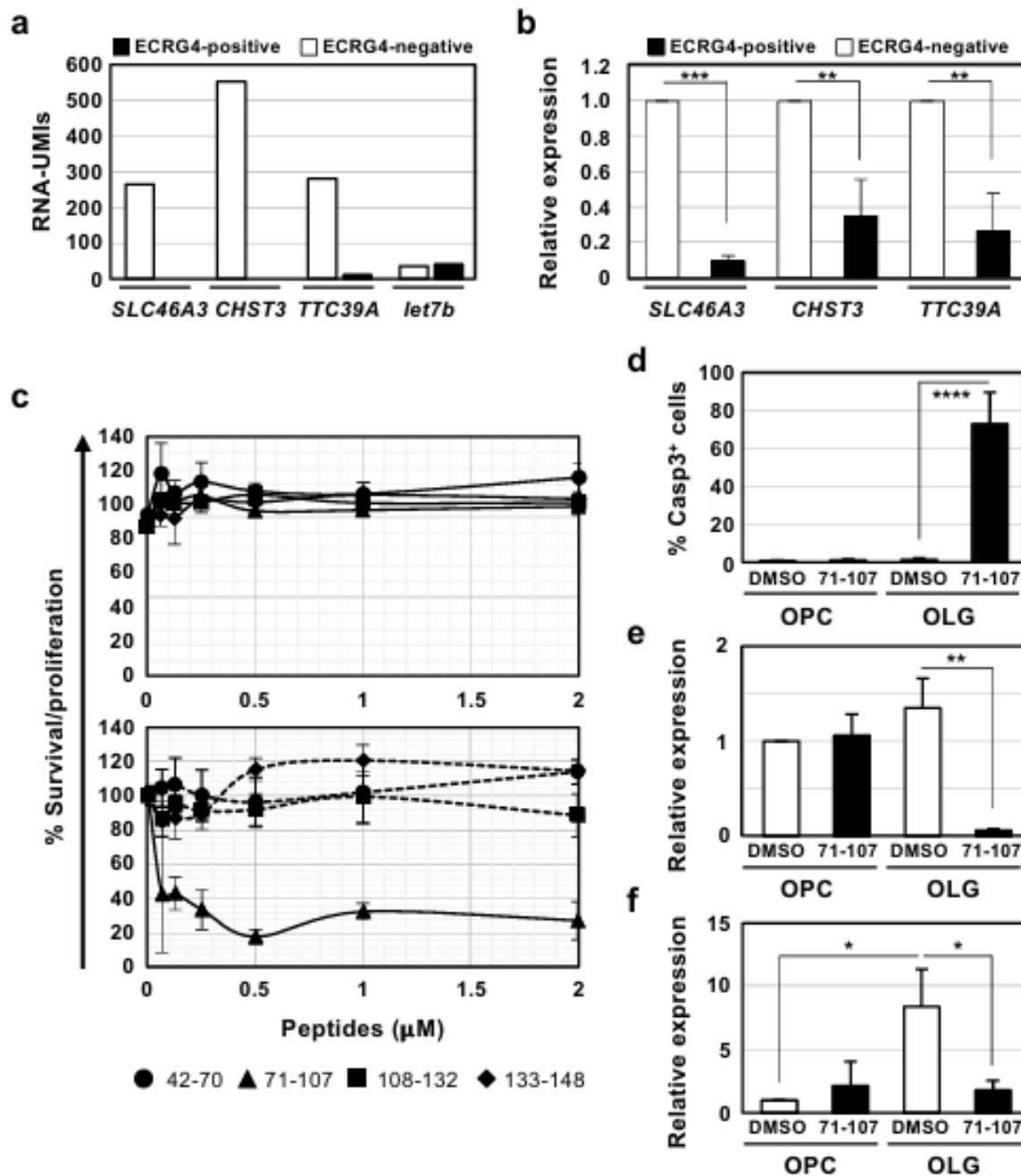


Fig. 11. Cytotoxicity of ECRG4 (71-107) to OLGs.

(a) Expression levels of three candidate genes, *SLC46A3*, *CHST3* and *TTC39A*, along with the control microRNA *let-7b* as determined by RNA seq analysis. UMIs: Unique Molecular Identifiers. (b) RT-qPCR measurement of *SLC46A3*, *CHST3* and *TTC39A* in ECRG4-positive and -negative plasma. (c) Proportion of proliferating/surviving neurons (upper figure) and oligodendrocyte (OLG) lineage cells (lower figure) cultured with a set of ECRG4 peptides, 42-70 (circle), 71-107 (triangle), 108-132 (square) and 133-148 (diamond). (d) Proportion of cleaved Casp3-positive cells, OPC and OLG, cultured with either DMSO alone or 71-107. (e) Relative *Chst3* expression in the cells, OPC and OLG, cultured with either DMSO alone or

71-107. (f) Relative *Chst3* mRNA in the supernatant of the cells, OPC and OLG, cultured with either DMSO alone or 71-107. Error bar: $\pm$ SD. Statistical significance was determined using an unpaired two-tailed *t*-test. **P* < 0.05, ***P* < 0.01, *** *p* < 0.001, **** *p* < 0.0001.

This is consistent with previous reports indicating that both neurons—especially GABAergic neurons—and OLGs are damaged in the brains of AD patients (Behrendt et al., 2013, Papuc and Rejdak, 2020, Temple, 2023, Zou et al., 2023). Therefore, I examined whether ECRG4 affects OLGs and neurons viability *in vitro*. Since ECRG4 is physiologically cleaved by proteases and present as fragments in extracellular space, I treated neurons and OLGs with different ECRG4 fragments, ECRG4 (42-70), ECRG4 (71-107), ECRG4 (108-132), and ECRG4 (133-148), that covers from N-terminus to C-terminus of released form of ECRG4 protein (Gonzalez et al., 2011, Qin and Zhang, 2019). Through MTT assay, I observed that neurons viability was not affected by any ECRG4 fragments. In contrast, the viability of OLGs decreased by ECRG4 (71-107) in concentration-dependent manner (Fig. 11c).

To further investigate the cytotoxicity of ECRG4 (71-107) on OLG lineage cells, I separately cultured OLGs and OPCs in the presence of the peptide and then immunostained the cells for cleaved Caspase 3 (Casp3), a marker of cell death (Fig. 12). ECRG4 (71-107) treatment did not increase the number of caspase-3 positive cells in OPCs while the fragment treatment significantly increased the number of caspase-3 positive cells in OLGs (Fig. 11d, Fig. 12). Additionally, I examined the effect of ECRG4 (71-107)-conditioned medium on the expression levels of *Chst3* mRNA both intracellularly and in the culture medium. The results demonstrated that ECRG4 (71-107) reduces *Chst3* mRNA expression in OLGs (Fig. 11e) as well as in the surrounding medium (Fig. 11f). Collectively, these data suggest that ECRG4 (71-107)-dependent cytotoxicity in OLGs leads to a decrease in plasma *CHST3* mRNA levels *in vitro*.

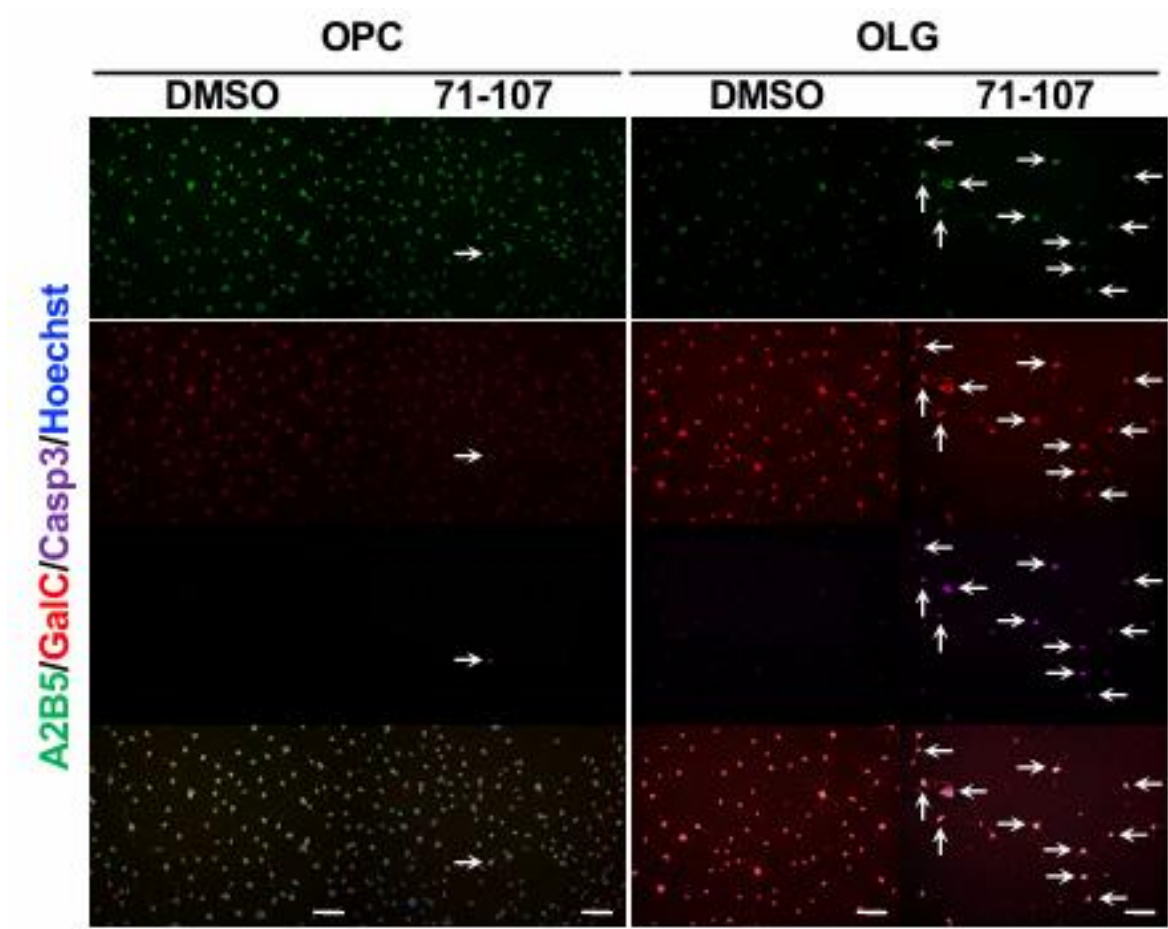


Fig. 12. The expression of caspase3 increased in OLGs under ECRG4 (71-107) culture conditions.

OPC and OLG were cultured in the presence of DMSO alone or ECRG4 (71-107) for three days, then immunolabeled with A2B5 (green) for OPC, anti-GalC (red) for OLG and anti-cleaved Casp3 (purple) for cell death. Arrows indicate Casp3-positive cells. Scale bar: 100 μ m.

ECRG4 affects OLGs, OPCs and vascular integrity *in vivo*.

Next, I explored how ECRG4 (71-107)-dependent OLG cytotoxicity increases the levels of the ECRG4 peptide in the plasma of AD patients. Niu et al. have shown that OPCs in the brains of multiple sclerosis (MS) model mice form clusters around the perivascular regions and increase vascular permeability by disrupting the BBB in a Wnt inhibitory factor 1 (Wif1)-dependent manner (Niu et al., 2019). I hypothesized that migrating OPCs, regenerating OLGs injured by ECRG4 (71-107), form perivascular clusters and compromise the BBB, similar to what is observed in the MS model.

To test this hypothesis, I injected ECRG4 (71-107) into the brains of mice five times in

total (Fig. 13a), prepared brain sections, and performed immunostaining for the OLG marker GalC, the endothelial cell marker CD31, the OPC marker PDGFR α , and mouse IgG to detect BBB leakage. As shown in Fig. 13b, c, peptide injection significantly decreased GalC intensity in the corpus callosum by approximately 47%, suggesting a reduction in myelin integrity and OLG numbers. Consistently, I observed a significant reduction (approximately 32%) in the level of Chst3 mRNA in the blood of peptide-injected mice (Fig. 13d), which correlates with the plasma results from AD patients (Fig. 11a,b).

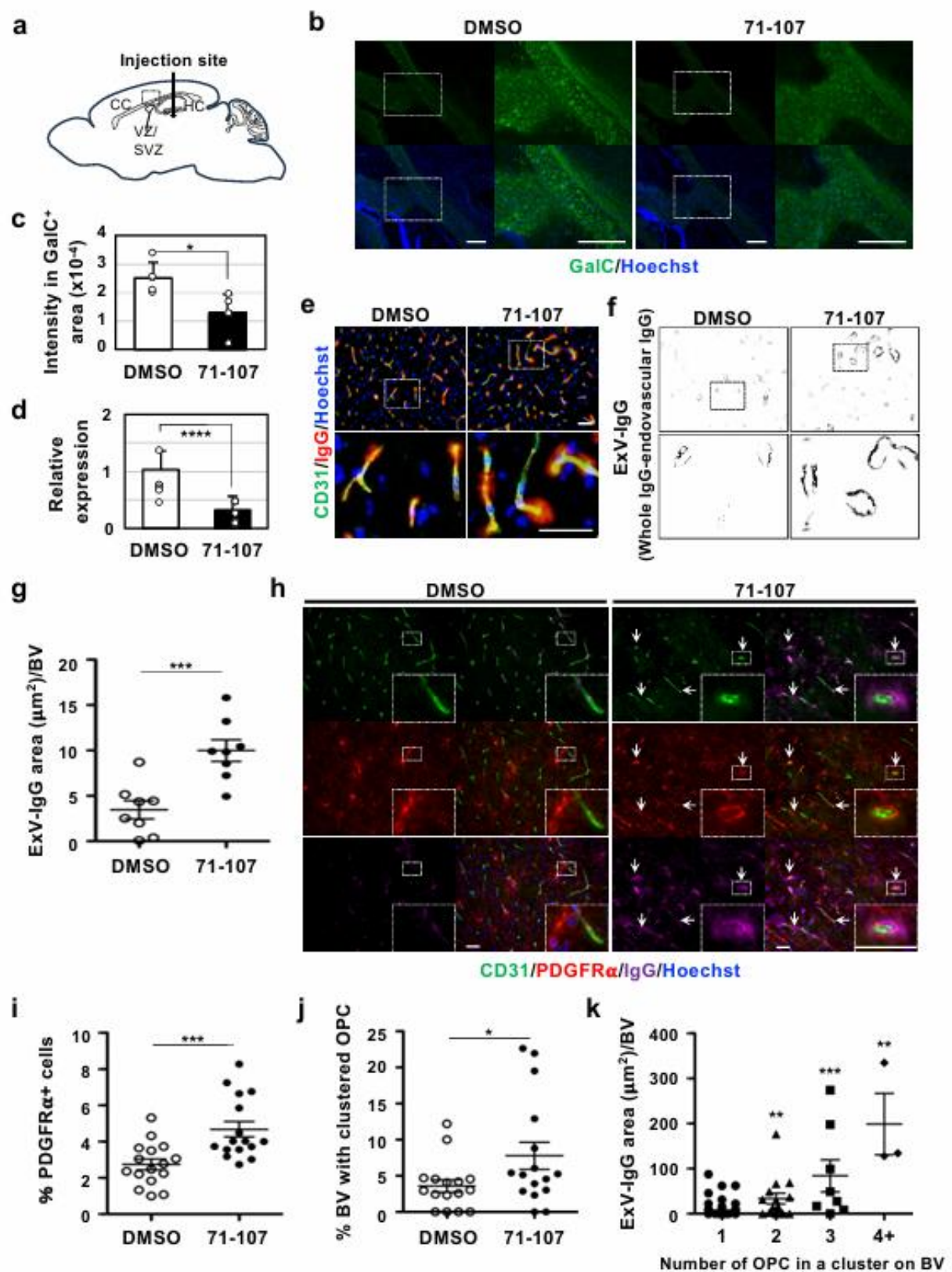


Fig. 13. Intracerebral injection of ECRG4 (71-107) decreased galactocerebroside and induced BBB leakage with OPC clustering on blood vessels.

(a) Schematic view of the ECRG4 (71-107) injection site. CC: corpus callosum, HC: hippocampus, VZ: ventricular zone, SVZ: subventricular zone.

hippocampus, VZ/SVZ: ventricular zone/subventricular zone. (b) Representative images of immunoreactivity for galactocerebroside (GalC, green) in brains injected with either DMSO alone (left panels) or ECRG4 (71-107) (right panels). (c) Quantitative intensity of GalC⁺ area in the corpus callosum injected with either DMSO alone or ECRG4 (71-107). (d) Relative level of *Chst3* mRNA in the plasma of mice injected with either DMSO alone or ECRG4 (71-107). (e) Representative images of immunoreactivity for the endothelial marker CD31 (green) and mIgG (red) in brains that were injected with either DMSO alone (left panels) or ECRG4 (71-107) (right panels). The lower panels show high magnification images of the boxed area outlined by a white dotted line in the upper panels. (f) Representative images of extravascular mouse IgG (ExV-IgG) generated by removing endovascular IgG from whole IgG. (g) Quantitative data of ExV-IgG area per blood vessel (BV). (h) Representative images of immunoreactivity for CD31 (green), an OPC maker PDGFR α (red) and mIgG (purple) in brains injected with either DMSO alone (left panels) or ECRG4 (71-107) (right panels). Arrows indicate the OPC cluster on BV. (i) Percentage of PDGFR α ⁺ cells shown in (h). (j) Percentage of BV with clustered OPC shown in (h). (k) Quantitative data of ExV-IgG area per number of OPC in a cluster on BV. All nuclei were counterstained with Hoechst 33342 (blue). Error bar: $\pm$ SD. Statistical significance was determined using an unpaired two-tailed *t*-test. **P* < 0.05, ***P* < 0.01, ****p* < 0.001, *****p* < 0.0001. Scale bar: 100 μ m.

Immunostaining revealed a significant amount of extravascular IgG (ExV-IgG) in the peptide-injected brains, indicating ECRG4 (71-107)-dependent BBB disruption (Fig. 13e-g). As expected, I observed that PDGFR α ⁺ OPCs not only increased in the peptide-injected brain but also formed clusters on the BV (Fig. 13h-j). Furthermore, the number of OPCs in a cluster on BV correlated with the amount of ExV-IgG (Fig. 3k). Together with an evidence that OPCs express *Wif1* with the peptide independently (data not shown), these data suggest that ECRG4 (71-107) dependent OLG cytotoxicity disrupts the BBB by clustering OPCs on BVs, leading to increased levels of ECRG4 peptides in the plasma of AD patients.

The increased number of OPCs and their clustering on BVs are phenomena that occurred selectively in EAD

To examine the dynamic state of OPCs in the brains of AD patients, I immunolabeled the hippocampal sections of patients with EAD, AD and non-dementia individuals, all of whom were APOE3/3 subtype, for the OPC marker NG2 and the astrocyte marker GFAP, and with UEA1 for detecting endothelial cells. My observations indicated that both the number of OPCs and their clusters on BVs increased in EAD as seen in the brains of ECRG4 (71-107)-injected mice (Fig. 13h-j). In contrast, the numbers of OPCs in AD patients were similar to those in non-dementia patients (Fig. 14a-c). Additionally, I found a significant decrease in GFAP⁺ astrocytes in AD, suggesting that astrocyte defects occur in the late phase of the disease. These data further support the notion that OPC clustering-dependent BBB breakdown facilitates the release of ECRG4 peptides into the blood stream during the early-stage of AD.

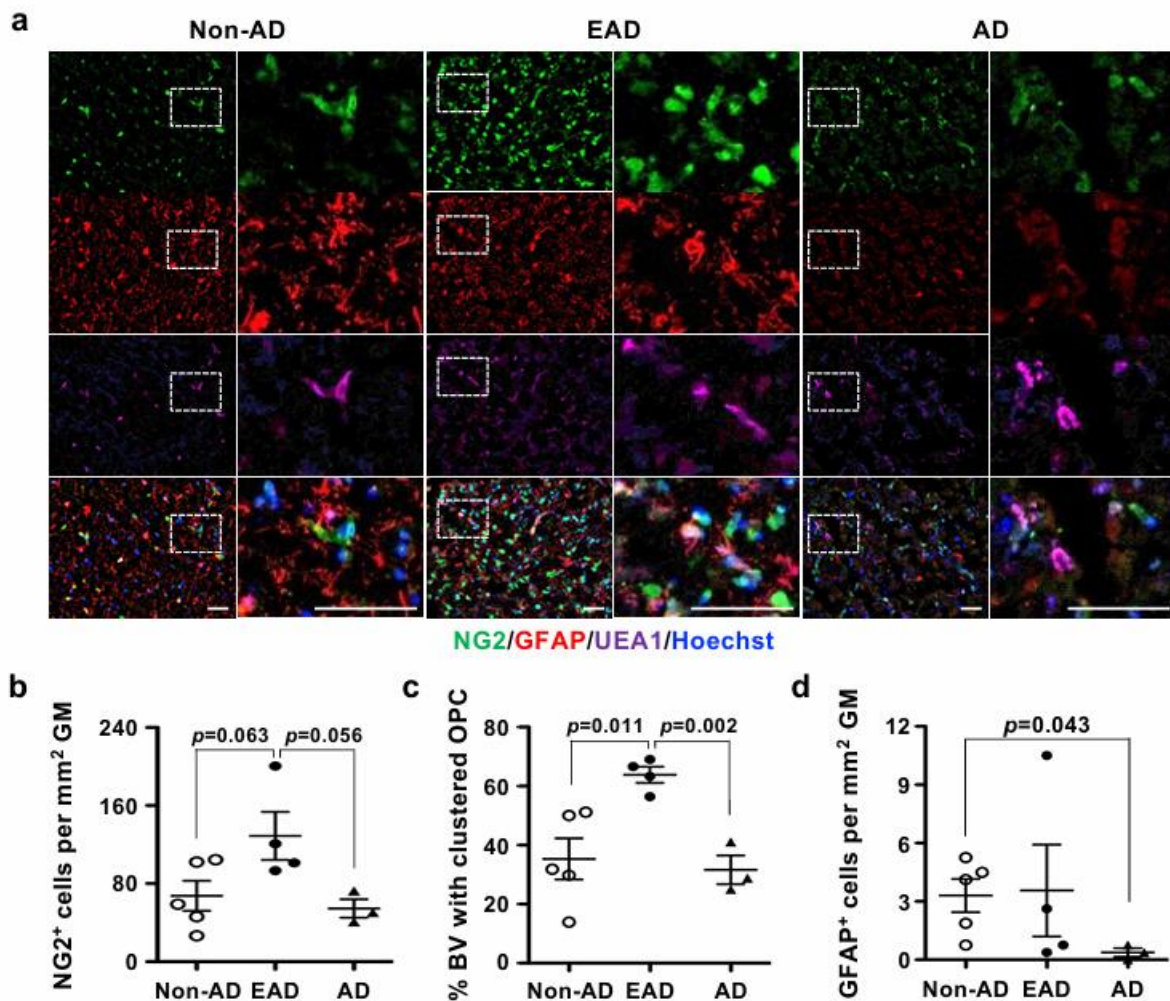


Fig. 14. OPCs transiently increase and form clusters on BV in the hippocampus of EAD.

(a) Representative images of immunoreactivity for the OPC marker NG2 (green), the astrocyte marker GFAP (red) and the endothelial specific UEA1 signal (purple) in the hippocampus of non-AD (left panels), EAD (middle panels) and AD (right panels). The right panels of each sample show high magnification images of the boxed area outlined by a white dotted line in the left panels. All nuclei were counterstained with Hoechst 33342 (blue). Scale bar: 100 μ m. (b) Number of NG2-positive cells per square mm in the gray matter of non-AD, EAD and AD. (c) Percentage of BV with clustered OPC. (d) Number of GFAP-positive cells per a square mm in gray matter of non-AD, EAD and AD. Error bar: $\pm$ SD. Statistical significance was determined using an unpaired two-tailed *t*-test.

Immunization of mice

In my ELISA system, I employed a mAb against ECRG4 as the capture Ab and a polyclonal Ab as the detection Ab. However, the production of polyclonal Abs is not sustainable. To prevent depletion of these Abs, I aim to generate a new monoclonal Ab and further engineer it into a chimeric Ab to improve my ELISA system (Phakham et al., 2022).

To achieve this goal, I will first produce new ECRG4 mAbs. To generate new mAbs, I first used ECRG4 as an antigen to immunize a group of mice. ELISA results showed that the Ab concentration in the mice's serum gradually increased over time following immunization (Fig. 15a). Additionally, I observed an enlargement of the spleen in the immunized mice (not shown), along with a noticeable increase in the size of their lymph nodes (Fig. 15b). These observations indicate that the prepared ECRG4 antigen successfully induced an immune response in the mice.

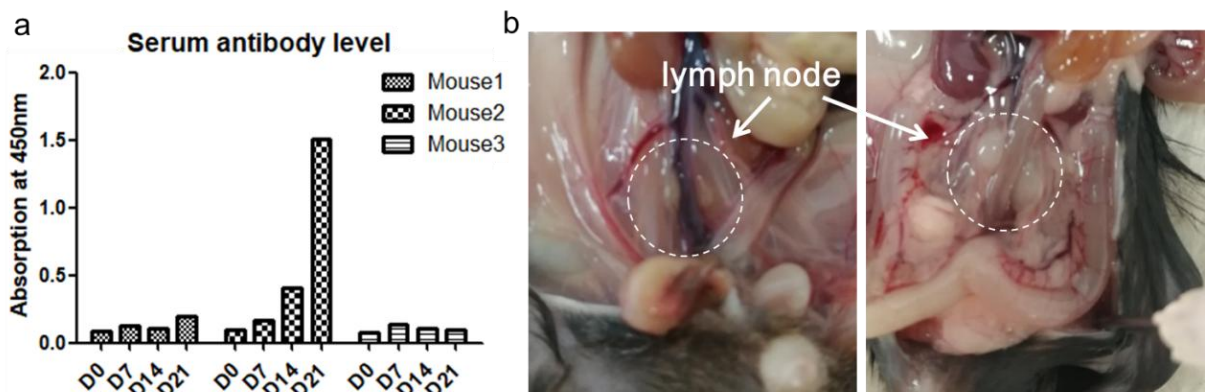


Fig. 15 Immunization effect in mice.

(a) A batch of three mice was immunized. The serum of the immunized mice was collected weekly and the Ab concentration in the serum was tested using the ELISA system. The higher

the absorbance, the higher the Ab concentration. (b) The left and right figures show the changes in lymph node size before and after immunization, respectively.

Cell fusion and screening

After cell fusion, I cultured the mixed cells in HAT medium. HAT medium is selection medium for cell culture. Aminopterin block DNA de novo synthesis pathway. Myeloma cells alone cannot proliferate in HAT medium. B cell dies as they have a short life. Only the fused cells of these two cells can proliferate (Fig. 16). I found that 528 of the 1920 wells had cell colony which mean cell proliferated. The probability of successful fusion of the two kinds of cells is 27.5%

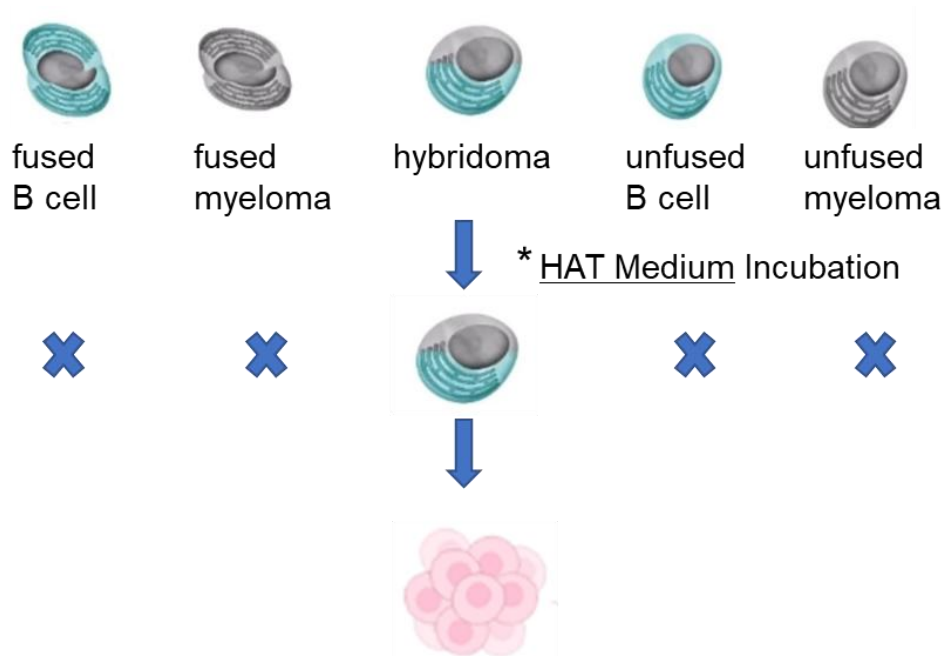


Fig. 16 Schematic diagram of HAT medium screening.

HAT (hypoxanthine-aminopterin-thymidine) Medium. Aminopterin is an inhibitor to block DNA de novo synthesis pathway. Hypoxanthine and thymidine are intermediates which could be used in DNA synthesis salvage pathway. B cells has de novo pathway and salvage pathway. Myeloma cells only has de novo pathway. Aminopterin in the medium blocks the de novo pathway. Hence, myeloma cells die, as they cannot produce DNA to proliferate by the de novo. B cells die as they have a short lifespan. Only the B cell-myeloma hybridoma can proliferate immortally.

Subsequently, I conducted the first screening of wells containing cell clusters. The

Ab concentration in the supernatant of the cell colonies was measured using ELISA (Fig. 17a). The results showed that out of 528 wells, 46 wells had an absorbance value greater than 0.2, and these wells were considered likely to contain Abs, accounting for approximately 8.71% (Fig. 17b).

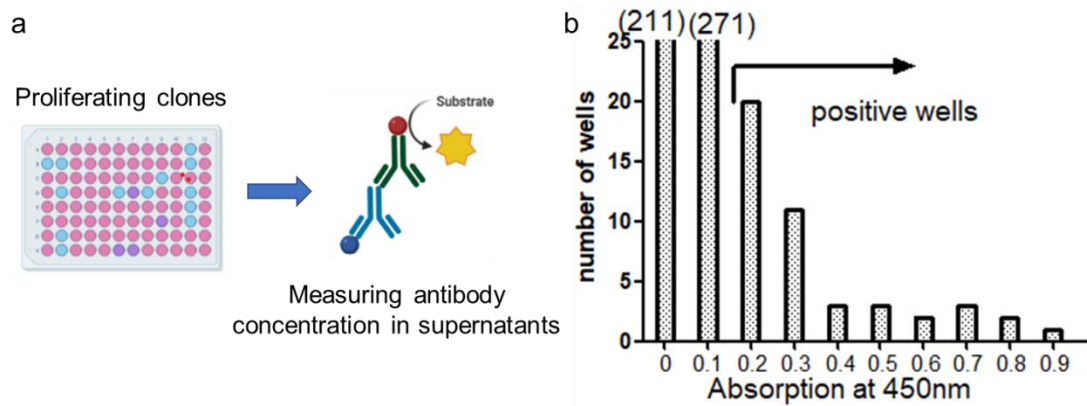


Fig. 17 Screening of hybridoma cells producing Abs.

(a) Schematic of the process for screening Ab-producing hybridoma cells. Collect the supernatant of clustered cells for ELISA. The absorbance is used to determine whether Abs are produced. (b) Statistical results of absorbance of each well. Wells with absorbance above 0.2 were considered to be positive for containing Abs.

Refining the produced Ab

After several rounds of screening, I successfully obtained stable Ab-secreting hybridoma cells. Using ProG beads to collect the mAbs from the supernatant (Fig. 18a), I analyzed the Ab content through SDS-PAGE. Since FCS is rich in IgG, in the medium containing 10% FCS, I observed the IgG heavy and light chains from the FCS binding to the ProG beads. In the medium with 2.5% FCS, the IgG heavy and light chain bands were also visible, but these bands appeared darker compared to those in the 10% FCS medium due to Ab production. Even in the medium without FCS (0% FCS), I still detected the IgG heavy and light chain bands (Fig. 18b), indicating that these bands were not from the IgG present in the supernatant, but rather from the Abs produced by the hybridoma cells.

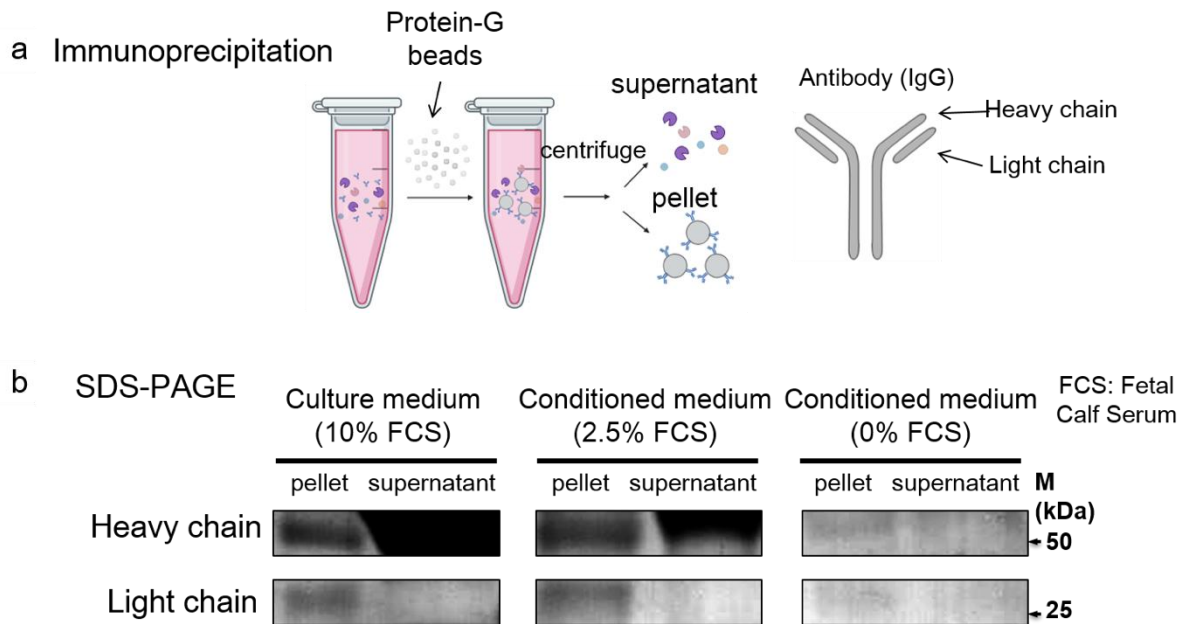


Fig. 18 The process of Ab refinement.

(a) The process of immunoprecipitation. The collected cell supernatant is mixed with ProG beads. The Ab (IgG) in the supernatant will bind to the ProG beads. The solution is centrifuged and the Ab-attached ProG beads are obtained. Abs (IgG) are composed of two light chains and two heavy chains. (b) SDS-PAGE was used to detect the presence of Abs. FCS medium also contains an amount of IgG. IgG on the beads was broken down into light chains and heavy chains by heating. Light chain and heavy chain bands can still be detected in the supernatant of medium without FCS.

Moving forward, I plan to isolate mRNA from the stable Ab-producing hybridoma cell lines and amplify and sequence the variable regions. Once the variable region sequences are identified, they will be combined with the constant regions from Abs of different species and expressed as recombinant chimeric Abs in an appropriate expression system. After establishing a new ELISA system using the chimeric Abs, I will be able to further investigate the relationship between ECRG4 levels in plasma and AD.

7. Discussion

ECRG4 serves as a sentinel molecule for monitoring tissue homeostasis and plays a critical role in the diagnosis and research of various diseases (Costantini et al., 2015, Huang et al., 2017, Wang and Ba, 2012). It is suggested that ECRG4 is cleaved by furin and thrombin. Our laboratory has previously demonstrated that the 71-132 fragment binds to various scavenger receptors, including the lectin-type oxidized LDL receptor 1 (LOX1) (Moriguchi et al., 2018). Additionally, Baird and colleagues reported that the 133-148 fragment binds to receptors such as toll-like receptor (TLR) 2/4 (Podvin et al., 2015). Both peptides have been shown to bind to receptors on microglia and macrophages, inducing the expression of inflammatory cytokines via nuclear factor kappa B (NF- κ B) pathway activation, as noted by both research teams.

While several molecular techniques, such as immunoblotting, immunohistochemistry, and quantitative RT-PCR, are available for detecting ECRG4, these methods are often time-consuming, labor-intensive, and require specific tissue samples, which limits their applicability in large-scale human sample analysis. Therefore, developing a simple, rapid, specific, sensitive, and efficient detection method—particularly a serological approach—is essential. In this context, my study developed an ECRG4 detection method based on ELISA, which allows for simultaneous analysis of a large number of samples using a 96-well plate. This significantly improves detection efficiency and makes it suitable for high-throughput screening (Whyte, 2024). ELISA is classified into several types, with the most common being direct ELISA and sandwich ELISA. I chose to use sandwich ELISA because it provides stronger signals, higher sensitivity, and greater specificity. The sandwich structure amplifies the signal, making it suitable for detecting low concentrations of proteins, while its higher specificity makes it ideal for complex samples (Thermofisher, 2024).

The ECRG4 ELISA system I developed has a detection limit (<30 pg/ml) can detect lower concentrations of ECRG4 peptides compared to commercially available kits (detection limit ≥ 30 pg/ml). Additionally, my system requires only half the sample volume used by other kits. Furthermore, my kit targets the 71-132 region, whereas the recognition region of commercial kits remains unspecified. Considering that ECRG4 can be cleaved by multiple proteases, with each resulting peptide potentially functioning as a bioactive factor, the development of kits to detect specific peptides will likely be necessary in the future. Thus, my ELISA system is considered superior to existing commercial kits. Regarding the

specificity of the antibody, I confirmed through WB and immunostaining that it does not bind to regions other than the ECRG4 antigen. Moreover, a similarity search using the peptide sequence employed to generate the 2A8 antibody revealed no hits other than ECRG4. These findings strongly suggest that the antibodies used in my system exhibit extremely high specificity. Given that ECRG4 can be cleaved by multiple proteases, each generating potentially bioactive peptides, the development of kits to detect specific peptides will likely be necessary in the future.

Analysis of clinical samples demonstrated that this sandwich ELISA system supported my initial hypothesis, indicating significantly elevated plasma levels of ECRG4 in patients with AD, which is strongly associated with AD pathology. This finding further underscores the diagnostic value of ECRG4 as a potential biomarker. However, it is worth noting that ECRG4 is also highly expressed in the plasma of patients with MCI and dementia symptoms, suggesting that ECRG4 may play a role in a shared pathogenic mechanism underlying these conditions. To validate the accuracy and broader applicability of this ELISA system, further studies with expanded clinical sample sets are needed to ensure its reliability in practical clinical pathological testing.

Interestingly, when comparing ECRG4 levels with classic AD biomarkers A β 40 and A β 42, I identified a novel pattern: while A β 40 and A β 42 levels were lower in AD patients, ECRG4 levels were elevated. Correlation analysis revealed a negative relationship between ECRG4 and A β 40, A β 42, and the A β 42/A β 40 ratio. Stratifying patients by gender showed significant differences in A β 40, A β 42, and the A β 42/A β 40 ratio in CSF, while ECRG4 exhibited pronounced differential expression in plasma, with no changes observed in CSF. This discrepancy could be due to different sources of ECRG4 in plasma and CSF, or it may reflect minimal fluctuations in CSF ECRG4 levels, which are insufficient for use as a diagnostic marker. I propose that ECRG4 may serve as a potential dementia-associated plasma biomarker. Compared to traditional CSF-based A β detection methods, plasma collection is minimally invasive. However, the specificity of ECRG4 detection remains a limitation, as not all patients with AD, MCI, or dementia exhibit detectable levels of ECRG4 in plasma. Further research is needed to understand the underlying mechanisms and its potential diagnostic value. Additionally, my study did not include the detection of phosphorylated Tau, as this requires either PET imaging or highly sensitive equipment capable of measuring trace amounts of phosphorylated Tau in blood. These methods do not align with my research objective of developing a simple and practical approach for the early diagnosis of AD. Therefore, phosphorylated Tau was not investigated in this study.

To investigate differences between patients in whom ECRG4 was detected in plasma (ECRG4+) and those in whom it was not (ECRG4-), I analyzed plasma RNA and identified three mRNAs that were decreased in ECRG4+ plasma. Among these mRNAs, two were expressed in inhibitory neurons and one in OLG lineage cells, which is consistent with prior reports of damage to these cell types in AD patients. This evidence suggests that the observed increase in plasma ECRG4 may stem from alterations in brain-resident cells.

OLGs are responsible for generating and maintaining myelin in the central nervous system, a structure that wraps around nerve fibers to enable rapid neural signal transmission and support proper neural signal conduction (Simons et al., 2024, Simons and Nave, 2016). OPCs, essential in the central nervous system, have the ability to differentiate into OLGs (Kirby et al., 2019).

In AD research, OLG dysfunction has been closely linked to myelin damage and neurodegeneration (Huang et al., 2024). The dysfunction and reduction of OLGs, which lead to myelin loss and subsequent neuronal injury, are recognized features of AD and other neurodegenerative disorders (Chen et al., 2023, Ettle et al., 2016, Papuc and Rejdak, 2020). There is evidence that impaired OLG function in AD may accelerate cognitive decline and neurodegenerative processes (Simons et al., 2023). Therefore, investigating gene expression changes in OLG lineage cells may provide further insight into the relationship between myelin abnormalities and neurodegeneration in AD pathology. Additionally, myelin loss may be linked to BBB dysfunction (Zou et al., 2023). Since ECRG4 is predominantly expressed in the brain (Gonzalez et al., 2011), impaired BBB function could allow ECRG4 to leak from the brain into the plasma, potentially contributing to elevated plasma levels of ECRG4.

In vitro experiments revealed that fragments of ECRG4 induced cell death in OLGs. I speculated that these ECRG4 fragments may bind to scavenger receptors on OLGs, activating innate immune responses and triggering cell death (Moriguchi et al., 2018), with this effect being dependent on the degree of cell differentiation. Following intracerebral injection of ECRG4 fragments into mice *in vivo*, I observed a reduction in GalC signal, vascular rupture, and accumulation of OPCs around BVs. These findings suggested that elevated ECRG4 expression in the brain may impair BBB function by affecting OLG lineage cells. However, whether ECRG4 peptides, including the 71-107 fragment, contribute to the onset of dementia remains unreported, and this is currently under investigation in our laboratory. Given prior reports that OPC perivascular accumulation due to OLG damage leads to BBB disruption, I concluded that the 71-107 fragment of ECRG4, produced by proteolytic cleavage in the brain, induces BBB disruption via OLG damage, subsequently causing ECRG4 peptides to leak into

the vasculature. This study was conducted by iteratively testing hypotheses based on RNA-seq data, publicly available gene expression information, and previous reports related to BBB disruption, representing a standard research approach.

In EAD patients, I observed OPC accumulation near BVs in the brain. Previous studies have noted that in MS, OPCs tend to cluster around BVs, losing their ability to migrate and thus failing to repair myelin (Zou et al., 2023). This observation aligns with what I noted in the brains of ECRG4-injected mice. I speculated that this clustering phenomenon may also be driven by ECRG4, as similar clustering was observed in experiments where ECRG4 was injected into the mouse brain. Additionally, I noted a decrease in GFAP signals in the brains of AD patients, suggesting a reduction in astrocytes at this stage, a critical component of the BBB (Manu et al., 2023). Combining these results, I hypothesized that the accumulation of OPCs around BVs may occur before the BBB is compromised, with ECRG4 as the driving force behind this accumulation.

I propose that during the early stages of AD, ECRG4 acts as a pro-inflammatory factor, with its expression increasing in the body (Moriguchi et al., 2016, Podvin et al., 2016). ECRG4 promotes OLG death and inhibits myelin repair mediated by OPCs, leading to neural demyelination. The loss or damage of myelin not only disrupts neural communication but also triggers a cascade of inflammatory responses (Rupnik et al., 2021). This inflammation, coupled with the accumulation of OPCs around BVs, can compromise the integrity of the BBB (Niu et al., 2019). The secretion of Wif1 and matrix metalloproteinase 9 (MMP9) by perivascular OPCs can disrupt the BBB, affecting astrocyte terminals and endothelial tight junctions, which leads to increased vascular permeability and central nervous system (CNS) inflammation. This may exacerbate neuronal injury and accelerate disease progression (Fang et al., 2023, Niu et al., 2019, Seo et al., 2013). Ultimately, the combined effects of OLG damage, myelin loss, and BBB dysfunction may create a vicious cycle that collectively promotes the development of neurodegenerative diseases (Zou et al., 2023).

This study has several limitations. First, the sample size, particularly for MCI, non-AD dementia, and healthy control groups, was relatively small and unevenly distributed across groups, which may limit statistical power and generalizability. Validation in larger, multicenter cohorts are warranted. Second, the use of patients with other neurological disorders as control subjects may introduce confounding factors, as these conditions could involve subtle BBB alterations or neuroinflammatory processes. Third, the specificity of the antibody against other proteins in actual samples has not been sufficiently validated, which may affect the reliability of the measured ECRG4 levels. Fourth, there is no direct evidence

demonstrating that plasma levels of the ECRG4 (108-132) fragment reflect the toxic effects of the ECRG4 (71–107) fragment in the brain. Finally, although our mechanistic findings provide insights into potential ECRG4-related pathways, they are preliminary and should be considered hypothesis-generating. As all experiments were conducted at a single institution using in-house reagents, further studies are needed to ensure reproducibility and consistency across different laboratories.

Future research should focus on determining whether alterations in plasma ECRG4 are specific to AD or indicative of a broader response to CNS injuries, such as cerebrovascular lesions or demyelinating disorders. These conditions may involve OLG damage or dysfunction of choroid plexus, which is known for its high expression of ECRG4.

In this study, I successfully completed the immunization of animals, hybridoma cell fusion, and the selection and validation of stable antibody-producing cell lines. These results provide valuable insights and foundational data for the future production of additional ECRG4 mAbs. However, several challenges and limitations were encountered during the process. For example, ECRG4 peptides may exhibit weak immunogenicity as antigens. To enhance the immune response, the peptide must be conjugated to a carrier protein, such as Keyhole limpet haemocyanin (KLH), prior to immunization (Ou et al., 2020). Additionally, administering only two antigen injections may not elicit a sufficient immune response; therefore, it is advisable to perform a booster injection three days before sacrificing the mice to maximize the immune response (Pettini et al., 2021). After sacrificing the mice, it would be essential to immerse the bodies in ethanol for five minutes to prevent contamination during the subsequent cell fusion experiments. This step may be crucial, as contamination could negatively impact the screening results.

8. Conclusion

My research shows:

1. ECRG4 is highly expressed in the plasma of AD and dementia patients.
2. The increased expression of ECRG4 in plasma is related to OLG lineage cells.
3. ECRG4 causes OLG death *in vitro*.
4. ECRG4 causes the reduction of CalC signal *in vivo*.
5. ECRG4 causes the accumulation of OPC on BVs and rupture it *in vivo*.
6. OPC clustering firstly occurs in the early stages of AD, followed by BV rupture and BBB damage.

These findings demonstrate that ECRG4 may be involved in the pathogenesis of AD and play a key role in myelin repair and BBB dysfunction. Significantly increased plasma ECRG4 is a potential biomarker for AD and dementia, highlighting its potential clinical significance.

However, the origins and underlying mechanisms of this ECRG4 elevation remain largely unresolved, posing several important questions. Firstly, the ELISA detection system used in this study specifically targets a defined region of the ECRG4 protein. It remains unclear if other regions of ECRG4 also exhibit changes in expression or post-translational modifications in plasma. This limitation could potentially restrict my understanding of ECRG4's role and dynamics in disease progression. Future improvements could involve utilizing alternative antibodies or advanced detection techniques to capture a more comprehensive profile of ECRG4 expression in plasma.

Additionally, while increased ECRG4 in plasma might suggest BBB impairment allowing ECRG4 leakage from the brain, it is possible that peripheral sources also contribute. For instance, increased levels of ECRG4 might stem from peripheral monocytes, as suggested by previous studies (e.g., Podvin, 2016). Disentangling these possible origins would require further investigation, possibly through analyses that compare ECRG4 levels in both CNS and peripheral immune cell populations.

The mechanisms driving ECRG4 upregulation also warrant exploration. The observed increase may result from elevated gene transcription, increased protein synthesis, or a reduction in degradation processes. Clarifying these pathways could provide insight into whether ECRG4 upregulation is a direct response to CNS pathology or secondary to systemic

inflammatory responses. Employing molecular analyses, such as qPCR for gene expression and proteomics for turnover rate studies, might yield valuable data on ECRG4 regulatory dynamics in AD and other dementias.

Furthermore, if BBB breakdown is indeed facilitating ECRG4 leakage into the bloodstream, theoretically, other brain-derived proteins, such as A β 40, would also exhibit increased plasma levels. However, my data did not show a parallel increase in A β 40, suggesting that BBB disruption might selectively impact protein transport or that specific mechanisms regulate ECRG4 differently. This finding raises the question of whether additional brain-derived proteins are also elevated in plasma and if so, what selective mechanisms might contribute to this phenomenon. Such an investigation could deepen my understanding of selective permeability changes in BBB dysfunction.

Moreover, my current histological analysis of human brain tissue has focused primarily on the hippocampus, a region critical to AD pathology. However, I have yet to examine ECRG4 levels in other brain regions, which could potentially offer insights into region-specific expression patterns or BBB integrity issues across the CNS. Future studies could expand to include cortical and subcortical areas, providing a more comprehensive understanding of ECRG4 distribution and its possible functional roles.

Finally, my findings show that ECRG4 is also elevated in patients with non-AD dementias, a result that suggests a potential role for ECRG4 beyond AD pathology. This broader expression profile prompts investigation into ECRG4's involvement in other forms of dementia and whether its mechanisms differ or overlap with those in AD. Future studies should aim to differentiate ECRG4's function across neurodegenerative conditions, examining the protein in varying dementia subtypes to clarify its broader diagnostic or prognostic value.

In conclusion, addressing these open questions through expanded experimental approaches will be essential to fully elucidate the role of ECRG4 in AD and other neurodegenerative diseases, and to explore its potential as a biomarker and therapeutic target.

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10. Disclosure of Conflict of Interest

The author declares no conflict of interest.

11. References

Albert MS, Dekosky ST, Dickson D, Dubois B, Feldman HH, Fox NC, Gamst A, Holtzman DM, Jagust WJ, Petersen RC, et al. (2011) The diagnosis of mild cognitive impairment due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers & Dementia*, 7, 270-279.

Alzheimer's & Dementia (2023) 2023 Alzheimer's disease facts and figures. *Alzheimer's and Dementia.*, 19, 1598-1695.

Arevalo-Rodriguez I, Smailagic N, Roque IFM, Ciapponi A, Sanchez-Perez E, Giannakou A, Pedraza OL, Bonfill Cosp X & Cullum S (2015) Mini-Mental State Examination (MMSE) for the detection of Alzheimer's disease and other dementias in people with mild cognitive impairment (MCI). *Cochrane Database Syst Rev*, 2015, CD010783.

Aschenbrenner AJ, Li Y, Henson RL, Volluz K, Hassenstab J, Verghese P, West T, Meyer MR, Kirmess KM, Fagan AM, et al. (2022) Comparison of plasma and CSF biomarkers in predicting cognitive decline. *Ann Clin Transl Neurol*, 9, 1739-1751.

Baird A, Coimbra R, Dang X, Lopez N, Lee J, Krzyzaniak M, Winfield R, Potenza B & Eliceiri BP (2012) Cell surface localization and release of the candidate tumor suppressor Ecrg4 from polymorphonuclear cells and monocytes activate macrophages. *J Leukoc Biol*, 91, 773-81.

Barres BA, Hart IK, Coles HSR, Burne JF, Voyvodic JT, Richardson WD & Raff MC (1992) Cell-Death in the Oligodendrocyte Lineage. *Journal of Neurobiology*, 23, 1221-1230.

Behrendt G, Baer K, Buffo A, Curtis MA, Faull RL, Rees MI, Gotz M & Dimou L (2013) Dynamic changes in myelin aberrations and oligodendrocyte generation in chronic amyloidosis in mice and men. *Glia*, 61, 273-86.

Bertram L, Lill CM & Tanzi RE (2010) The genetics of Alzheimer disease: back to the future. *Neuron*, 68, 270-81.

Bomasang-Layno E & Bronsther R (2021) Diagnosis and Treatment of Alzheimer's Disease:: An Update. *Dela J Public Health*, 7, 74-85.

Brand AL, Lawler PE, Bollinger JG, Li Y, Schindler SE, Li M, Lopez S, Ovod V, Nakamura A, Shaw LM, et al. (2022) The performance of plasma amyloid beta measurements in identifying amyloid plaques in Alzheimer's disease: a literature review. *Alzheimers Res Ther*, 14, 195.

Chen GF, Xu TH, Yan Y, Zhou YR, Jiang Y, Melcher K & Xu HE (2017) Amyloid beta: structure, biology and structure-based therapeutic development. *Acta Pharmacol Sin*, 38, 1205-1235.

Chen P, Guo Z & Zhou B (2023) Disease-Associated Oligodendrocyte: New Player in Alzheimer's Disease and CNS Pathologies. *J Integr Neurosci*, 22, 90.

Chung J, Zhang X, Allen M, Wang X, Ma Y, Beecham G, Montine TJ, Younkin SG, Dickson DW, Golde TE, et al. (2018) Genome-wide pleiotropy analysis of neuropathological traits related to Alzheimer's disease. *Alzheimers Res Ther*, 10, 22.

Costantini TW, Coimbra R, Lopez NE, Lee JG, Potenza B, Smith A, Baird A & Eliceiri BP (2015) Monitoring Neutrophil-Expressed Cell Surface Esophageal Cancer Related Gene-4 after Severe Burn Injury. *Surg Infect (Larchmt)*, 16, 669-74.

Dang X, Podvin S, Coimbra R, Eliceiri B & Baird A (2012) Cell-specific processing and release of the hormone-like precursor and candidate tumor suppressor gene product, Ecrg4. *Cell Tissue Res*, 348, 505-14.

Donato R, Miljan EA, Hines SJ, Aouabdi S, Pollock K, Patel S, Edwards FA & Sinden JD (2007) Differential development of neuronal physiological responsiveness in two human neural stem cell lines. *BMC Neurosci*, 8, 36.

Ettle B, Schlachetzki JCM & Winkler J (2016) Oligodendroglia and Myelin in Neurodegenerative Diseases: More Than Just Bystanders? *Mol Neurobiol*, 53, 3046-3062.

Fang TN, Dai YQ, Hu XY, Xu YH & Qiao JP (2024) Evaluation of serum neurofilament light chain and glial fibrillary acidic protein in the diagnosis of Alzheimer's disease. *Frontiers in Neurology*, 15.

Fang YC, Hsieh YC, Hu CJ & Tu YK (2023) Endothelial Dysfunction in Neurodegenerative Diseases. *Int J Mol Sci*, 24.

Fillenbaum GG & Mohs R (2023) CERAD (Consortium to Establish a Registry for Alzheimer's Disease) Neuropsychology Assessment Battery: 35 Years and Counting. *J Alzheimers Dis*, 93, 1-27.

Folstein MF, Folstein SE & Mchugh PR (1975) Mini-Mental State - Practical Method for Grading Cognitive State of Patients for Clinician. *Journal of Psychiatric Research*, 12, 189-198.

Gallwitz M, Enoksson M, Thorpe M & Hellman L (2012) The Extended Cleavage Specificity of Human Thrombin. *Plos One*, 7.

Gonzalez AM, Podvin S, Lin SY, Miller MC, Botfield H, Leadbeater WE, Robertson A, Dang X, Knowling SE, Cardenas-Galindo E, et al. (2011) Ecrg4 expression and its product

augurin in the choroid plexus: impact on fetal brain development, cerebrospinal fluid homeostasis and neuroprogenitor cell response to CNS injury. *Fluids Barriers CNS*, 8, 6.

Gotze S, Feldhaus V, Traska T, Wolter M, Reifemberger G, Tannapfel A, Kuhnen C, Martin D, Muller O & Sievers S (2009) ECRG4 is a candidate tumor suppressor gene frequently hypermethylated in colorectal carcinoma and glioma. *BMC Cancer*, 9, 447.

Gustavsson A, Norton N, Fast T, Frolich L, Georges J, Holzapfel D, Kirabali T, Krolak-Salmon P, Rossini PM, Ferretti MT, et al. (2023) Global estimates on the number of persons across the Alzheimer's disease continuum. *Alzheimers Dement*, 19, 658-670.

Hansson O, Zetterberg H, Buchhave P, Andreasson U, Londos E, Minthon L & Blennow K (2007) Prediction of Alzheimer's disease using the CSF A β 42/A β 40 ratio in patients with mild cognitive impairment. *Dementia and Geriatric Cognitive Disorders*, 23, 316-320.

Harada R, Ishiki A, Kai H, Sato N, Furukawa K, Furumoto S, Tago T, Tomita N, Watanuki S, Hiraoka K, et al. (2018) Correlations of (18)F-THK5351 PET with Postmortem Burden of Tau and Astroglisis in Alzheimer Disease. *J Nucl Med*, 59, 671-674.

Hide T, Takezaki T, Nakatani Y, Nakamura H, Kuratsu J & Kondo T (2009) Sox11 Prevents Tumorigenesis of Glioma-Initiating Cells by Inducing Neuronal Differentiation. *Cancer Research*, 69, 7953-7959.

Huang L, Yu H, Fan X, Li X, Mao L, Cheng J, Zeng X & Dang X (2017) A Potential Role of Esophageal Cancer Related Gene-4 for Atrial Fibrillation. *Sci Rep*, 7, 2717.

Huang Z, Jordan JD & Zhang Q (2024) Myelin Pathology in Alzheimer's Disease: Potential Therapeutic Opportunities. *Aging Dis*, 15, 698-713.

Ingannato A, Bagnoli S, Mazzeo S, Giacomucci G, Bessi V, Ferrari C, Sorbi S & Nacmias B (2024) Plasma GFAP, NfL and pTau 181 detect preclinical stages of dementia. *Front Endocrinol (Lausanne)*, 15, 1375302.

Jack CR, Jr. & Holtzman DM (2013) Biomarker modeling of Alzheimer's disease. *Neuron*, 80, 1347-58.

Kaufer DI, Williams CS, Braaten AJ, Gill K, Zimmerman S & Sloane PD (2008) Cognitive Screening for Dementia and Mild Cognitive Impairment in Assisted Living: Comparison of 3 Tests. *Journal of the American Medical Directors Association*, 9, 586-593.

Kirby L, Jin J, Cardona JG, Smith MD, Martin KA, Wang JY, Strasburger H, Herbst L, Alexis M, Karnell J, et al. (2019) Oligodendrocyte precursor cells present antigen and are cytotoxic targets in inflammatory demyelination. *Nature Communications*, 10.

Kizuka Y, Kitazume S, Fujinawa R, Saito T, Iwata N, Saido TC, Nakano M, Yamaguchi Y, Hashimoto Y, Staufenbiel M, et al. (2015) An aberrant sugar modification of BACE1

blocks its lysosomal targeting in Alzheimer's disease. *EMBO Mol Med*, 7, 175-89.

Klyucherev TO, Olszewski P, Shalimova AA, Chubarev VN, Tarasov VV, Attwood MM, Syvanen S & Schioth HB (2022) Advances in the development of new biomarkers for Alzheimer's disease. *Transl Neurodegener*, 11, 25.

Kujuro Y, Suzuki N & Kondo T (2010) Esophageal cancer-related gene 4 is a secreted inducer of cell senescence expressed by aged CNS precursor cells. *Proc Natl Acad Sci U S A*, 107, 8259-64.

Lee J, Dang X, Borboa A, Coimbra R, Baird A & Eliceiri BP (2015) Thrombin-processed EcrG4 recruits myeloid cells and induces antitumorigenic inflammation. *Neuro Oncol*, 17, 685-96.

Li LW, Yu XY, Yang Y, Zhang CP, Guo LP & Lu SH (2009) Expression of esophageal cancer related gene 4 (ECRG4), a novel tumor suppressor gene, in esophageal cancer and its inhibitory effect on the tumor growth in vitro and in vivo. *Int J Cancer*, 125, 1505-13.

Lian H, Roy E & Zheng H (2016) Protocol for Primary Microglial Culture Preparation. *Bio Protoc*, 6.

Logovinsky V, Satlin A, Lai R, Swanson C, Kaplow J, Osswald G, Basun H & Lannfelt L (2016) Safety and tolerability of BAN2401--a clinical study in Alzheimer's disease with a protofibril selective Abeta antibody. *Alzheimers Res Ther*, 8, 14.

Ma LN, Wang R, Han Y, Sheng SL, Zhu JA, Ji ZJ, Zhao ZW, Cao ZQ & Wang PC (2016) Development of a Novel Urine Alzheimer-Associated Neuronal Thread Protein ELISA Kit and Its Potential Use in the Diagnosis of Alzheimer's Disease. *Journal of Clinical Laboratory Analysis*, 30, 308-314.

Manu DR, Slevin M, Barcutean L, Forro T, Boghitoiu T & Balasa R (2023) Astrocyte Involvement in Blood-Brain Barrier Function: A Critical Update Highlighting Novel, Complex, Neurovascular Interactions. *Int J Mol Sci*, 24.

Matsuzaki J, Torigoe T, Hirohashi Y, Kamiguchi K, Tamura Y, Tsukahara T, Kubo T, Takahashi A, Nakazawa E, Saka E, et al. (2012) ECRG4 is a negative regulator of caspase-8-mediated apoptosis in human T-leukemia cells. *Carcinogenesis*, 33, 996-1003.

Mbl. How to generate antibodies [Online]. Available: <https://ruo.mbl.co.jp/bio/e/support/method/antibody-production.html> [Accessed 11 November 2024].

Mbl. How to select antibodies [Online]. Available: <https://ruo.mbl.co.jp/bio/e/support/method/antibody-select.html> [Accessed 11 November 2024].

Menck K, Bleckmann A, Schulz M, Ries L & Binder C (2017) Isolation and Characterization of Microvesicles from Peripheral Blood. *J Vis Exp*.

Mirabeau O, Perlas E, Severini C, Audero E, Gascuel O, Possenti R, Birney E, Rosenthal N & Gross C (2007) Identification of novel peptide hormones in the human proteome by hidden Markov model screening. *Genome Res*, 17, 320-7.

Mori H, Hosoda K, Matsubara E, Nakamoto T, Furiya Y, Endoh R, Usami M, Shoji M, Maruyama S & Hirai S (1995) Tau in cerebrospinal fluids: establishment of the sandwich ELISA with antibody specific to the repeat sequence in tau. *Neurosci Lett*, 186, 181-3.

Moriguchi T, Kaneumi S, Takeda S, Enomoto K, Mishra SK, Miki T, Koshimizu U, Kitamura H & Kondo T (2016) *Ecr4* contributes to the anti-glioma immunosurveillance through type-I interferon signaling. *Oncoimmunology*, 5, e1242547.

Moriguchi T, Takeda S, Iwashita S, Enomoto K, Sawamura T, Koshimizu U & Kondo T (2018) *Ecr4* peptide is the ligand of multiple scavenger receptors. *Sci Rep*, 8, 4048.

Nakamura A, Kaneko N, Villemagne VL, Kato T, Doecke J, Doré V, Fowler C, Li QX, Martins R, Rowe C, et al. (2018) High performance plasma amyloid- β biomarkers for Alzheimer's disease. *Nature*, 554, 249-+.

Nakatani Y, Kiyonari H & Kondo T (2019) *Ecr4* deficiency extends the replicative capacity of neural stem cells in a *Foxg1*-dependent manner. *Development*, 146.

Nishide K, Nakatani Y, Kiyonari H & Kondo T (2009) Glioblastoma formation from cell population depleted of *Prominin1*-expressing cells. *PLoS One*, 4, e6869.

Niu J, Tsai HH, Hoi KK, Huang N, Yu G, Kim K, Baranzini SE, Xiao L, Chan JR & Fancy SPJ (2019) Aberrant oligodendroglial-vascular interactions disrupt the blood-brain barrier, triggering CNS inflammation. *Nat Neurosci*, 22, 709-718.

Ou L, Kong WP, Chuang GY, Ghosh M, Gulla K, O'dell S, Varriale J, Barefoot N, Changela A, Chao CW, et al. (2020) Preclinical Development of a Fusion Peptide Conjugate as an HIV Vaccine Immunogen. *Scientific Reports*, 10.

Ow SY & Dunstan DE (2014) A brief overview of amyloids and Alzheimer's disease. *Protein Science*, 23, 1315-1331.

Ozawa A, Lick AN & Lindberg I (2011) Processing of proaugurin is required to suppress proliferation of tumor cell lines. *Mol Endocrinol*, 25, 776-84.

Papuc E & Rejdak K (2020) The role of myelin damage in Alzheimer's disease pathology. *Arch Med Sci*, 16, 345-351.

Pettini E, Pastore G, Fiorino F, Medagliani D & Ciabattini A (2021) Short or Long Interval between Priming and Boosting: Does It Impact on the Vaccine Immunogenicity?

Vaccines, 9.

Phakham T, Boonkrai C, Wongtangprasert T, Audomsun T, Attakitbanha C, Saelao P, Muanwien P, Sooksai S, Hirankarn N & Pisitkun T (2022) Highly efficient hybridoma generation and screening strategy for anti-PD-1 monoclonal antibody development. *Scientific Reports*, 12.

Podvin S, Dang X, Meads M, Kurabi A, Costantini T, Eliceiri BP, Baird A & Coimbra R (2015) Esophageal cancer-related gene-4 (ECRG4) interactions with the innate immunity receptor complex. *Inflamm Res*, 64, 107-18.

Podvin S, Miller MC, Rossi R, Chukwueke J, Donahue JE, Johanson CE, Baird A & Stopa EG (2016) The Orphan C2orf40 Gene is a Neuroimmune Factor in Alzheimer's Disease. *JSM Alzheimers Dis Relat Dement*, 3.

Porzionato A, Rucinski M, Macchi V, Sarasin G, Malendowicz LK & De Caro R (2015) ECRG4 expression in normal rat tissues: expression study and literature review. *Eur J Histochem*, 59, 2458.

Qin X & Zhang P (2019) ECRG4: a new potential target in precision medicine. *Front Med*, 13, 540-546.

Querfurth HW & Laferla FM (2010a) Alzheimer's disease. *N Engl J Med*, 362, 329-44.

Querfurth HW & Laferla FM (2010b) MECHANISMS OF DISEASE Alzheimer's Disease. *New England Journal of Medicine*, 362, 329-344.

Rashad A, Rasool A, Shaheryar M, Sarfraz A, Sarfraz Z, Robles-Velasco K & Cherrez-Ojeda I (2022) Donanemab for Alzheimer's Disease: A Systematic Review of Clinical Trials. *Healthcare (Basel)*, 11.

Rupnik M, Baker D & Selwood DL (2021) Oligodendrocytes, BK channels and the preservation of myelin. *F1000Res*, 10, 781.

Sado Y, Inoue S, Tomono Y & Omori H (2006) Lymphocytes from enlarged iliac lymph nodes as fusion partners for the production of monoclonal antibodies after a single tail base immunization attempt. *Acta Histochem Cytochem*, 39, 89-94.

Saxton J, Morrow L, Eschman A, Archer G, Luther J & Zuccolotto A (2009) Computer Assessment of Mild Cognitive Impairment. *Postgraduate Medicine*, 121, 177-185.

Schindler SE, Bollinger JG, Ovod V, Mawuenyega KG, Li Y, Gordon BA, Holtzman DM, Morris JC, Benzinger TLS, Xiong C, et al. (2019) High-precision plasma beta-amyloid 42/40 predicts current and future brain amyloidosis. *Neurology*, 93, e1647-e1659.

Seo JH, Miyamoto N, Hayakawa K, Pham LD, Maki T, Ayata C, Kim KW, Lo EH & Arai K (2013) Oligodendrocyte precursors induce early blood-brain barrier opening after

white matter injury. *J Clin Invest*, 123, 782-6.

Sevigny J, Chiao P, Bussiere T, Weinreb PH, Williams L, Maier M, Dunstan R, Salloway S, Chen T, Ling Y, et al. (2016) The antibody aducanumab reduces Abeta plaques in Alzheimer's disease. *Nature*, 537, 50-6.

Shinohara M, Hirokawa J, Shimodaira A, Tashiro Y, Suzuki K, Gheni G, Fukumori A, Matsubara T, Morishima M, Saito Y, et al. (2021) ELISA Evaluation of Tau Accumulation in the Brains of Patients with Alzheimer Disease. *Journal of Neuropathology and Experimental Neurology*, 80, 652-662.

Shoji M & Kanai M (2001) Cerebrospinal fluid Abeta40 and Abeta42: Natural course and clinical usefulness. *J Alzheimers Dis*, 3, 313-321.

Simons M, Gibson EM & Nave KA (2024) Oligodendrocytes: Myelination, Plasticity, and Axonal Support. *Cold Spring Harb Perspect Biol*.

Simons M, Levin J & Dichgans M (2023) Tipping points in neurodegeneration. *Neuron*, 111, 2954-2968.

Simons M & Nave KA (2016) Oligodendrocytes: Myelination and Axonal Support. *Cold Spring Harbor Perspectives in Biology*, 8.

Takanaga H, Tsuchida-Straeten N, Nishide K, Watanabe A, Aburatani H & Kondo T (2009) Gli2 Is a Novel Regulator of Sox2 Expression in Telencephalic Neuroepithelial Cells. *Stem Cells*, 27, 165-174.

Tarawneh R & Holtzman DM (2012) The clinical problem of symptomatic Alzheimer disease and mild cognitive impairment. *Cold Spring Harb Perspect Med*, 2, a006148.

Temple S (2023) Advancing cell therapy for neurodegenerative diseases. *Cell Stem Cell*, 30, 512-529.

ThermoFisher. Overview of ELISA [Online]. Available: <https://www.thermoFisher.com/jp/ja/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html> [Accessed 11 November 2024].

ThermoFisher (2024) ELISA vs Western Blot: When to Use Each Immunoassay Technique.

Wang YB & Ba CF (2012) Promoter methylation of esophageal cancer-related gene 4 in gastric cancer tissue and its clinical significance. *Hepatogastroenterology*, 59, 1696-8.

Waybright T, Avellino AM, Ellenbogen RG, Hollinger BJ, Veenstra TD & Morrison RS (2010) Characterization of the human ventricular cerebrospinal fluid proteome obtained from hydrocephalic patients. *J Proteomics*, 73, 1156-62.

Whyte B. (2024) Optimizing your ELISA Assays [Online]. Available: <https://www.bmglabtech.com/en/blog/optimizing-your-elisa-assays/> [Accessed 11 November 2024].

Woo JM, Park SJ, Kang HI, Kim BG, Shim SB, Jee SW, Lee SH, Sin JS, Bae CJ, Jang MK, et al. (2010) Characterization of changes in global gene expression in the brain of neuron-specific enolase/human Tau23 transgenic mice in response to overexpression of Tau protein. *International Journal of Molecular Medicine*, 25, 667-675.

Xu PT, Li YJ, Qin XJ, Kroner C, Green-Odlum A, Xu H, Wang TY, Schmechel DE, Hulette CM, Ervin J, et al. (2007) A SAGE study of apolipoprotein E3/3, E3/4 and E4/4 allele-specific gene expression in hippocampus in Alzheimer disease. *Mol Cell Neurosci*, 36, 313-31.

Yue CM, Deng DJ, Bi MX, Guo LP & Lu SH (2003) Expression of ECRG4, a novel esophageal cancer-related gene, downregulated by CpG island hypermethylation in human esophageal squamous cell carcinoma. *World J Gastroenterol*, 9, 1174-8.

Zlokovic BV (2011) Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. *Nat Rev Neurosci*, 12, 723-38.

Zou P, Wu C, Liu TC, Duan R & Yang L (2023) Oligodendrocyte progenitor cells in Alzheimer's disease: from physiology to pathology. *Transl Neurodegener*, 12, 52.