科技部補助專題研究計畫成果報告
期末報告

第一型類胰島素生長因子訊息途徑保護神經細胞對抗乙型類澱粉蛋白毒性之機轉研究(第3年)

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中 華 民 國 104 年 10 月 30 日

中 文 摘 要 ： 阿滋海默症(Alzheimer's disease)是一種最常見的神經退化性疾病，臨床上主要具備兩種病理特徵，包括在神經細胞外由Aβ所聚而成的類澱粉(amyloid plaques)及在細胞內由過度磷酸化的tau所組成神經纖維糾結(neurofibrillary tangles)等。越來越多的證據指出阿滋海默症基本上是屬於代謝疾病，事實上臨床與實驗上的結果皆顯示阿滋海默症腦部的神經細胞其胰島素生長因子的訊息傳遞均有不正常受到抑制的情形。這些結果顯示了胰島素的訊息抵抗可能是連結代謝症候群與阿滋海默症兩者間的共同分子機轉，然而胰島素的訊息傳遞與Aβ造成神經退化之間的確切關聯則不是非常清楚，然而越來越多的證據指出當周邊系統發生代謝紊亂時，可能會因此一併導致腦部的胰島素訊息亦發生阻抗的情況，而這種現象被認為與Aβ在腦部的神經毒性反應有關。由於在AD的致病過程中Aβ始終扮演著核心角色，因此我們認為Aβ可能是透過惡化腦部胰島素訊息阻抗的方式，來增強其神經毒性並導致神經退化。根據我們的研究結果，我們發現 (i)神經胰島素訊息傳遞與Aβ神經毒性之間有直接的相關性，同時也能透過提升胰島素訊息活性來對抗Aβ神經毒性；(2)透過提升AMPK活性除可減緩Aβ所造成的神經胰島素阻抗外，亦可活化細胞自噬作用而增加神經細胞在逆境下的生存率；(3)幹細胞因子Nanog會參與神經胰島素訊息傳遞的調控，並因此發揮神經保護的功效。總結來說，我們認為本研究結果有助於釐清腦部胰島素訊息阻抗在AD病程進展中所扮演的角色，並提出可以利用Nanog來調控胰島素訊息傳遞並對抗AD的新機制，未來將可能據此發展出可減緩或中斷AD病程進展的新型治療策略。

中 文 關 鍵 詞 ： 阿滋海默症、β類澱粉蛋白、胰島素訊息、單磷酸線苷活化激酶、神經保護

英 文 摘 要 ： Alzheimer's disease (AD) is the most common neurodegenerative disease. It is characterized by two diagnostic pathological hallmarks like amyloid plaques (APs) which are mainly formed by Aβ peptides accumulation, and neurofibrillary tangles (NFTs) which are composed of hyperphosphorylated tau protein. Growing evidence supports the concept that AD is fundamentally a metabolic disease. In fact, both clinical and experimental data demonstrated insulin signaling was aberrantly distributed in AD neurons. Consequently, neuronal resistance for insulin signaling might represent a molecular link between metabolic syndrome and AD. However, the mechanism how insulin signaling influences the onset and progression of Aβ-induced neurodegeneration remain incompletely understood. However, increasing evidence supports that AD is fundamentally a metabolic disorders that leads to brain insulin resistance. This indicates AD and type 2 diabetes mellitus (T2DM) may share some a common background in pathogenesis. As the Aβ plays a core role in the pathogenesis of AD, we postulated that Aβ might further deepen the progress of brain insulin resistance and contribute to its neurotoxicity. According

to our results, we found that (i) there is a significant correlation between neuronal insulin signaling and Aβ-induced neurotoxicity, and the stimulation of insulin signaling could alleviate Aβ-induced neurotoxicity; (ii) AMPK activation not only alleviates Aβ-induced neuronal insulin resistance, and contributes to neuronal survival under stress by autophagy induction; (iii) the stem cell-related factor Nanog was indeed involved in the insulin signaling regulation, and displayed protective effects against neurotoxicity of Aβ. In conclusion, our results can be a contribution to the of the understanding mechanisms involved in neuronal insulin signaling, and shed light onto possible therapeutic approaches to provide new AD therapeutic strategies and drug targets in future.

科技部補助專題研究計畫成果報告

（□期中進度報告/☑期末報告）

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中　華　民　國 104 年 10 月 31 日

中文摘要

阿滋海默症(Alzheimer's disease)是一種最常見的神經退化性疾病,臨床上主要具備兩種病理特徵,包括在神經細胞外由 Aβ 所聚而成的類澱粉(amyloid plaques)及在細胞內由過度磷酸化的 tau 所組成神經纖維糾結(neurofibrillary tangles)等。越來越多的證據指出阿滋海默症基本上是屬於代謝疾病,事實上臨床與實驗上的結果皆顯示阿滋海默症腦部的神經細胞其胰島素生長因子的訊息傳遞均有不正常受到抑制的情形。這些結果顯示了胰島素的訊息抵抗可能是連結代謝症候群與阿滋海默症兩者間的共同分子機轉,然而胰島素的訊息傳遞與 Aβ 造成神經退化之間的確切關聯則不是非常清楚,然而越來越多的證據指出當周邊系統發生代謝紊亂時,可能會因此一併導致腦部的胰島素訊息亦發生阻抗的情況,而這種現象被認為與 Aβ 在腦部的神經毒性反應有關。由於在 AD 的致病過程中 Aβ 始終扮演著核心角色,因此我們認為 Aβ 可能是透過惡化腦部胰島素訊息阻抗的方式,來增強其神經毒性並導致神經退化。根據我們的研究結果,我們發現 (i)神經胰島素訊息傳遞與 Aβ 神經毒性之間有直接的相關性,同時也能透過提升胰島素訊息活性來對抗 Aβ 神經毒性;(2)透過提升 AMPK 活性除可減緩 Aβ 所造成的神經胰島素阻抗外,亦可活化細胞自噬作用而增加神經細胞在逆境下的生存率;(3)幹細胞因子 Nanog 會參與神經胰島素訊息傳遞的調控,並因此發揮神經保護的功效。總結來說,我們認為本研究結果有助於釐清腦部胰島素訊息阻抗在 AD 病程進展中所扮演的角色,並提出可以利用 Nanog 來調控胰島素訊息傳遞並對抗 AD 的新機制,未來將可能據此發展出可減緩或中斷 AD 病程進展的新型治療策略。

關鍵詞:阿滋海默症、β 類澱粉蛋白、胰島素訊息、單磷酸線苷活化激酶、神經保護

Abstract

Alzheimer's disease (AD) is the most common neurodegenerative disease. It is characterized by two diagnostic pathological hallmarks like amyloid plaques (APs) which are mainly formed by Aβ peptides accumulation, and neurofibrillary tangles (NFTs) which are composed of hyperphosphorylated tau protein. Growing evidence supports the concept that AD is fundamentally a metabolic disease. In fact, both clinical and experimental data demonstrated insulin signaling was aberrantly distributed in AD neurons. Consequently, neuronal resistance for insulin signaling might represent a molecular link between metabolic syndrome and AD. However, the mechanism how insulin signaling influences the onset and progression of Aβ-induced neurodegeneration remain incompletely understood. However, increasing evidence supports that AD is fundamentally a metabolic disorders that leads to brain insulin resistance. This indicates AD and type 2 diabetes mellitus (T2DM) may share some a common background in pathogenesis. As the Aβ plays a core role in the pathogenesis of AD, we postulated that Aβ might further deepen the progress of brain insulin resistance and contribute to its neurotoxicity. According to our results, we found that (i) there is a significant correlation between neuronal insulin signaling and Aβ-induced neurotoxicity, and the stimulation of insulin signaling could alleviate Aβ-induced neurotoxicity; (ii) AMPK activation not only alleviates Aβ-induced neuronal insulin resistance, and contributes to neuronal survival under stress by autophagy induction; (iii) the stem cell-related factor Nanog was indeed involved in the insulin signaling regulation, and displayed protective effects against neurotoxicity of Aβ. In conclusion, our results can be a contribution to the of the understanding mechanisms involved in neuronal insulin signaling, and shed light onto possible therapeutic approaches to provide new AD therapeutic strategies and drug targets in future.

Keywords: Alzheimer's disease, β amyloid, insulin signaling, AMP-activated protein kinase, neuroprotection

前言

　　根據行政院經建會推估,到 2011 年底台灣地區 65 歲以上老年人口將佔全國人口的 10.4%,因此台灣已漸漸面臨人口快速的老齡化的問題,如何解決老化相關的各類疾病,將成為今後醫學和社會上極重要的課題。其中,在老年人口的疾病當中,失智症(dementia),特別是阿茲海默氏症(Alzheimer's disease,簡稱 AD)最為大家所關注。根據統計,僅有少於 5﹪的 AD 患者其發病原因是由於某些基因或蛋白質突變,如 APP、presenilin-1 及 presenilin-2 等變異而導致,另外超過 95%的 AD 患者則是由其他尚未明瞭的病因所造成(sporadic AD),其原因可能是由於腦內代謝異常或訊息傳遞不正常所引發。研究發現,肥胖與心血管疾病均會顯著提高 AD 的罹患率,而肥胖與心血管疾病事實上皆與現今所謂的代謝徵候群(metabolic syndrome)或糖尿病高度相關。我們都知道糖尿病可分為兩型,第一型糖尿病是胰臟無法正常生產 insulin 所致,而第二型糖尿病的病人的體內雖然會生產 insulin,卻因為 insulin signaling 有問題,身體內各種組織對 insulin 無法起正常反應所致。在 sporadic AD 病人中隨著逐漸老化,腦中接受 insulin 訊號的效率開始越來越差,最終會誘發 Aβ 不正常的累積並加重其毒性反應。其實流行病學早有研究顯示,糖尿病人罹患 AD 的風險比一般人口高得多,另也有許多研究顯示,胰島素 (insulin)顯然有助減少腦部遭到 Aβ 的傷害。此外,許多預防糖尿病的方法都被證實具有預防 AD 的作用,同時糖尿病用藥也確實有減緩 AD 的效用,因此近年來,有些學者已提出新的說法,認為 AD 應屬於第三型糖尿病,其病因乃是腦中的 insulin/insulin-like growth factor 1 receptor 或 downstream signaling 受到阻礙而使然。已知腦內的 insulin signaling pathway 會調節大腦認知功能,包括學習和記憶等等。在人類和動物實驗都已證實 insulin 有增進記憶的作用。例如,給予被動逃避訓練經驗後(passive avoidance training experience),將 insulin 注入大鼠的第三腦室內,會有較佳的記憶存留(memory retention) 。此外,insulin signaling 和突觸可塑性(synaptic plasticity)及短期記憶形成有關,但確實的分子機制仍不清楚。在腦部中,突觸也是發生 insulin signaling pathway 的重要場所,因此,評估 insulin signaling 的效率可能可以作為神經元促進 synaptic plasticity 與 LTP,進而增進短期記憶有正面的關係。當 insulin 與其 receptor 連結後,會和 insulin receptor substrate (IRS)組成複合體,並促使 phosphatidylinositol 3-kinase (PI3K)磷酸化,進而活化 serine/threonine kinase Akt 以及其下游的細胞生理反應。眾所皆知,罹患 AD 最大的危險因子便是老化本身,因此若能釐清 insulin signaling 在神經細胞中的生理機制,將有助於瞭解 AD 的病程與老化之間的關聯性。因此,我們認為若能詳細探討神經細胞內 insulin 訊息傳遞調控的機轉,將有助於未來發展新型的 AD 疾病預防或治療策略。

目前在臨床上發展 AD 的治療方法大致上可分為兩個方向,包括症狀緩解策略(symptomatic strategies)及機制性治療策略(mechanism-based strategies),針對這些發展當前的狀況簡述如下:

(1)增加神經傳導物質:已知乙醯膽鹼(acetylcholine)為一種神經傳導物質,它與人

類記憶及認知能力有很大的關聯，若其合成及釋放的機制發生缺失，則會引起神經退化。而先前的研究已明確指出 AD 患者腦中乙醯膽鹼的含量較一般正常低，因此目前臨床上主要的治療策略便是利用乙醯膽鹼酶抑制劑(acetylcholinesterase inhibitors)來增加其含量，或是利用調控乙醯膽鹼的前驅物(acetylcholine precursors)來增加其含量等，以彌補 AD 患者所降低的乙醯膽鹼神經傳導物質，進而達到減緩症狀的目的。

(2)降低神經興奮性毒性(excitotoxicity)：所謂神經興奮性毒性是指的是神經細胞遭受 glutamate 或是相似物如 NMDA 的刺激之後，腦中的 NMDA receptor 和 AMPA receptor 可以和其結合，並促使 Ca^{2+} 流入細胞內破壞離子平衡而產生神經毒性。已知在 AD 患者的神經細胞附近可觀察到 glutamate 量增加或是 glutamate transporter 下降，使得 glutamate 無法被吸收回去，這些過量的 glutamate 便會活化神經興奮性毒性，進一步加重 AD 的症狀。Memantine 即是目前臨床上用來抑制此種神經興奮性毒性的藥物，其可以透過拮抗 NMDA 接受器，抑制過多的 glutamate 來減少興奮性毒性對神經的傷害。目前 memantine 主要用於已經有持續穩定使用 cholinesterase inhibitor 的中重度患者，其可以使病患認知能力改善，減緩病患日常活動力退化，並減少行為不正常的新症狀出現。但截至目前為止，基於以上增加神經傳導物質及降低神經興奮性毒性此兩點發展的藥物都沒有達到在根本上治癒 AD 的效果，所有的治療只是在保留或是改善其認知功能、減少行為混亂，並延緩疾病惡化。

(3)機制性策略(mechanism-based strategies)：機制性策略是基於 amyloid cascade hypothesis 的基礎，透過抑制 Aβ 的生成途徑(例如發展 β-secretase 與 γ-secretase 抑制劑等)，或清除腦內已產生之 Aβ (例如發展以 $Aβ_{1-42}$ 作為抗原的疫苗，藉由其所引起的免疫反應，來達到清除 Aβ 的目的)，或減低 Aβ 所產生的不良後果(例如利用 NSAIDs 減少 Aβ 在腦部所造成的發炎反應，以及蔡立慧院士透過調控 CDK5 來減輕 Aβ 的毒性反應等)。雖然這些發展中的藥物或治療方法或因缺乏實證醫學的數據，其療效目前仍有待確認，但歸根究底還是必須持續致力於發展機制性的策略，才能徹底解決 AD 在臨床上治療的問題。

研究目的

AD 是最常見的老年型失智症，面臨台灣即將到來的高齡化社會，AD 勢必成為未來重大的醫療問題。AD 患者腦部有兩個重要的病理指標，分別是 senile plaques 和 neurofibrillary tangles，其中前者主要由 Aβ 蛋白組成，而後者則由 tau 蛋白所構成。依目前的觀念，Aβ 會於 AD 的早期階段在患者腦部逐漸累積，隨著病程的進展並伴隨中後期所誘發的 tau 蛋白過度磷酸化，最終導致神經退化而引起失智的症狀，因此 Aβ 被認為是早期引發 AD 的主要根源。目前臨床上治療 AD 主要有兩個方式，包括利用 acetylcholinesterase inhibitors 來增加腦內神經傳導物質的濃度(e.g. rivastigmine)，或是給予 NMDA receptor antagonist 來降低神經興奮性毒性(e.g. memantine)，但這兩種機制其實都沒有針對 Aβ 所造成的問題進

行治療,是故仍然必須發展新的機制性策略,才有辦法在根本上解決 AD 的問題。關於這點,已有越來越多的證據指出第二型糖尿病(type 2 diabetes mellitus, T2DM)同時罹患阿茲海默症 (Alzheimer's disease, AD) 的機率會顯著提高,而 insulin/insulin growth factor (IGF)-1 signaling 被認為在其中可能扮演著關鍵性的角色,因此近年來有學者認為 AD 的病因乃是源自於腦中的 insulin signaling 受到阻礙而產生,但其與 Aβ 神經毒性之間的相互影響機制目前仍不明。而根據我們初步的實驗結果,發現 Aβ 會阻礙神經細胞內的 insulin signaling,並因此加重 Aβ 導致之神經毒性。據此我們提出一個假設,認為 Aβ 可經由誘發神經細胞的 insulin resistance 來導致神經退化,因此只要能設法改善 Aβ 所造成 insulin signaling 抑制的情況,便有機會在機制上減緩 AD 的病程進展。

實驗結果

在最近的研究中,我們與美國 WJWU & LYNN Institute for Stem Cell Research 之林希龍教授進行合作,建立 miR302 誘導 SK-N-MC 逆轉老化的細胞實驗模式。林希龍教授在先前的研究已指出 miR302 具有誘使細胞逆分化的趨勢,會使受誘導的細胞表現出類似幹細胞的特徵。在以下的實驗中,我們利用 miR302 誘導 SK-N-MC 逆轉老化,發現當細胞被誘發成類幹細胞的狀態時,會透過提升 Nanog 表現量來抑制 Aβ 所誘發的胰島素阻抗,並因此減緩 Aβ 所導致的神經傷害。基於這些初步實驗的結果,再加上 Aβ 被認為與 brain insulin resistance 有關,因此我們認為 Nanog 與 AD 疾病進展可能有所關聯。我們現已將 Sox-2、Oct-4、Klf-4 與 Nanog 四個基因的質體構築好並轉殖入神經細胞中,發現確實具有更顯著的抗 Aβ 神經毒性的能力,但仍待更深入的研究才能證實其機轉。
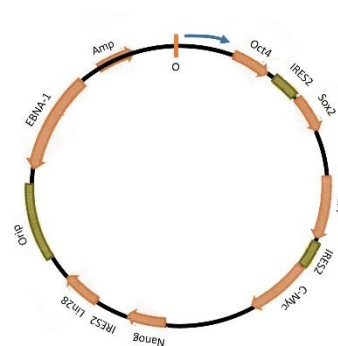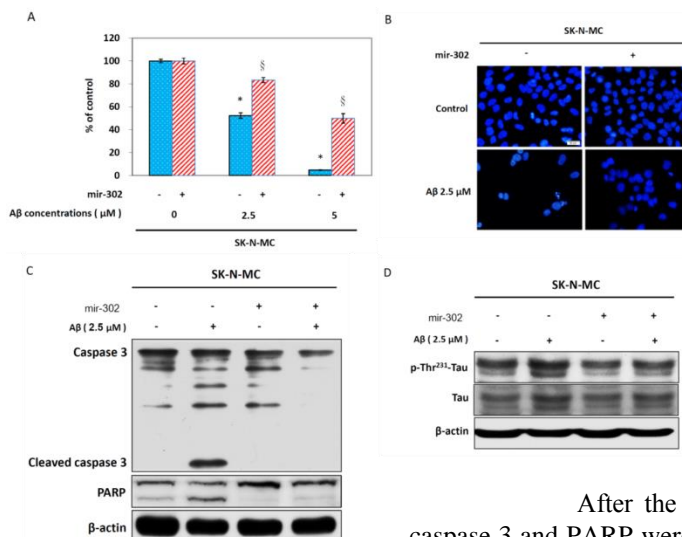

1. Generation of mir-302s overexpressed cell displays stem cell phenotypic markers
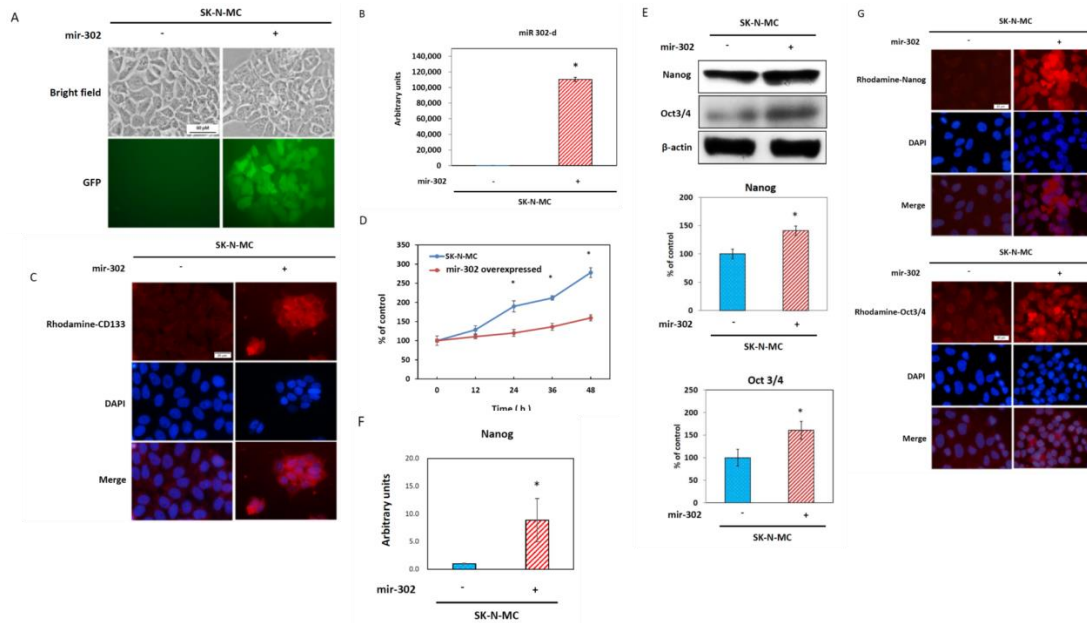
Nanog and Oct 3/4 transcription factors are correlates with expression of mir-302 miRNA (A) SK-N-MC cell were transfected with mir-302s plasmid, we used 1μg/ml puromycin selection cells until stable state. the stable state were detect GPF expression by fluorescence microscopy. (B, F) The stable states were harvested, and the RNA and protein were extracted from whole-cell lysates, which were analyzed by Real-time PCR with primers specific to mir-302d and Nanog, (E) by western blotting with antibodies specific to Nanog, Oct3/4 and β-actin. (G) by immunofluorescence with antibodies specific to Nanog, Oct3/4 and DAPI. (C) Further, we also detected the stem cell marker, CD133, by immunofluorescence and the induced the stem cell effect could be sustained for puromycin selection. (D) We detected the specific cell feature, cell growth curve, by cell counts for 0, 12, 24, 36 and 48 h after puromycin selection. (+, present; -, absent. Bar depict mean ± S.D. of at least three independent experiments. " * " represent a statistically significant difference from control group or the same group, *p*<0.05. " § " represent a statistically significant difference from different group treat with β , *p*<0.05)

2. mir-302s overexpressed cells attenuated Aβ-induced neurotoxicity

The overexpression of mir-302s inhibits exogenous Aβ-induced neurotoxicity. (A) Cells were treated with Aβ 2.5-5μM for 24 h by MTT assays. The cell survival rates were significantly decreased in SK-N-MC cells comparing with mir-302s overexpressed cells after treating Aβ dose of 2.5 and 5μM for 24 hr. (B) The morphological change of nuclear chromatin in apoptosis observed by fluorescent microscopy after Aβ treating for 24 hr. (C) Aβ-induced cell apoptosis wer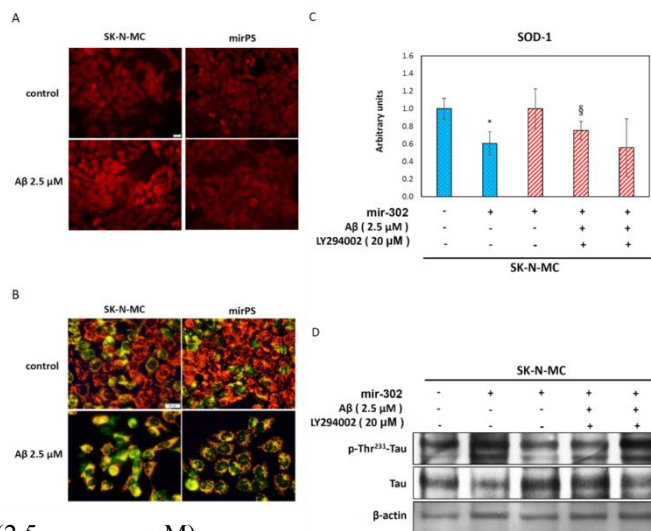e detected the protein levels expression by western blotting. After the treatment of Aβ 2.5 μM for 24 h, the caspase 3 and PARP were increased the expression in SK-N-MC cells comparing with mir-302s overexpressed cells. (D) Aβ-induced neurotoxicity were observed AD feature of the tau hyperphosphorylation by western blotting assays after the treatment of Aβ 2.5 μM for 24 hr. In SK-N-MC cells, Aβ-induced increased tau hyperphosphorylation comparing in mir-302s overexpressed cell. (+, present; -, absent. Bar depict mean ± S.D. of at least three independent experiments. " * " represent a statistically significant difference from control group or the same group, p<0.05. " § " represent a statistically significant difference from different group treat with β , p<0.05)

3. Activation of Akt/ GSK3β partially contributes to the anti-neurotoxicity role of mir-302s overexpression
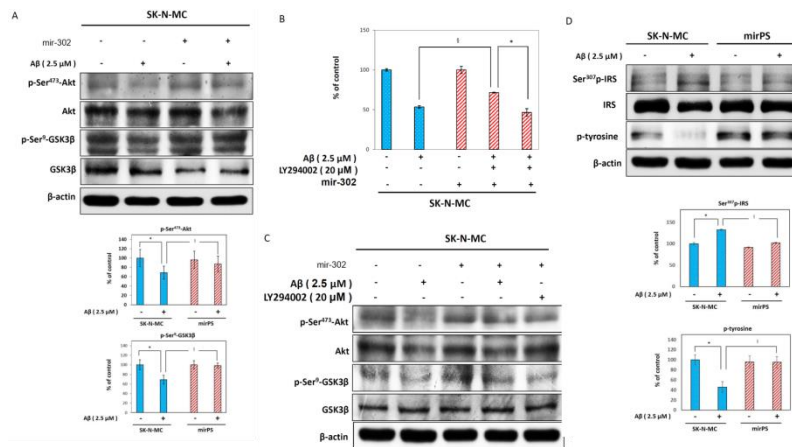
Activation of IRS-1/Akt/GSK3β against Aβ-induced neurotoxicity (A) The expressions of mir-302s mediated activation of IRS-1/Akt/GSK3β signaling after treatment of Aβ 2.5 μM for 24 h by western blotting assays. (B) Co-treated with Aβ (2.5 μM) and LY294002 (20 μM) were administered in mir-302s overexpressed cell incubation for 24 h, which cell viabilities were assayed by MTT assays. Comparing with only treatment of Aβ (2.5 μM), co-treatment were significantly decreased cell survival in mir-302s overexpressed cells. (C) Further, the protein levels of the p-Akt, Akt, p-GSK3β, GSK3β, p-IRS-1, IRS-1 and tyrosine were detected by western blotting. Co-treatment of Aβ (2.5 μM) and LY294002 (20 μM) decreased the protein level of p-Akt and p-GSK3β in mir-302s overexpressed cell, which were compared with only treatment of Aβ (2.5 μM). (D) Therefore, the protein level of p-IRS-1, IRS-1 and tyrosine were also affected after Co-treatment of Aβ and LY294002 in mir-302s overexpressed cell. (+, present; -, absent. Bar depict mean ± S.D. of at least three independent experiments. " * " represent a statistically significant difference from control group or the same group, p<0.05. " § " represent a statistically significant difference from different group treat with β , p<0.05)

4. Aβ implicated Akt signaling induces features of neurotoxicity
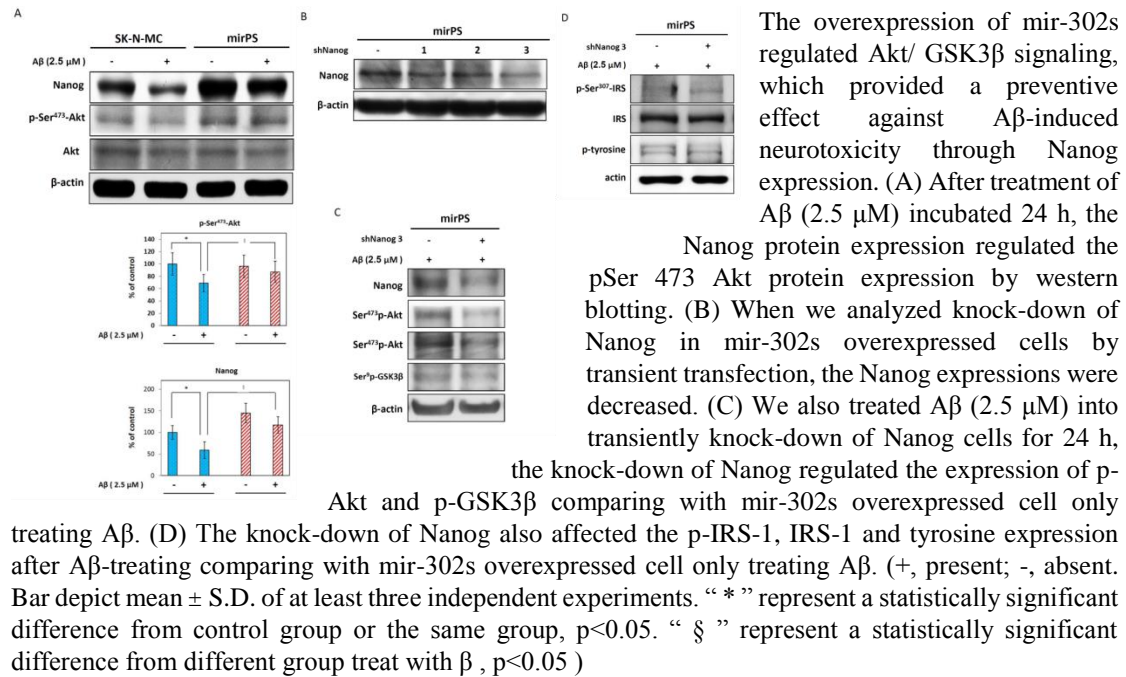

The overexpression of mir-302s inhibits Aβ-induced neurotoxicity. The expression of mir-302s attenuated Aβ-induced intracellular oxidative stress, mitochondrial dysfunction and tau hyperphosphorylation, which (A) intracellular superoxide radical anions were stained using hydroethideine stain by fluorescence microscopy. After Aβ (2.5 μM) incubated for 2hr, the red fluorescence were detected in SK-N-MC comparing with mir-302s overexpressed cells. (B) The mitochondrial membrane potential were stained using JC-1 dye by fluorescence microscopy. After Aβ (2.5 μM) incubated for 2 h, the SK-N-MC cells were apparent increased green monomer fluorescence comparing with non-treatment and mir-302s overexpressed cells treating with Aβ. (C) The mRNA levels of SOD-1 were detect by real time PCR, the SK-N-MC was decreased SOD-1 mRNA expression after Aβ treating, and comparing with the mir-302s overexpressed cells. (D) Aβ-induced neurotoxicity were also assayed tau hyperphosphorylation by western blotting after treatment of Aβ (2.5 μM) or LY294002 incubated for 24 hr. Co-treatment of Aβ
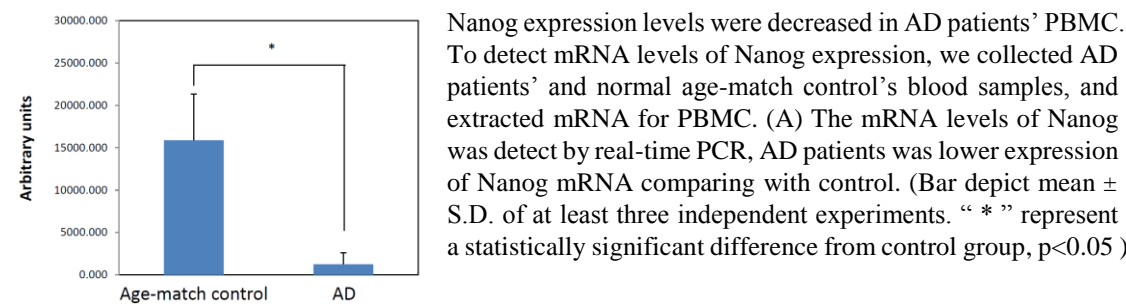
and LY294002 were increased tau hyperphosphorylation in mir-302s overexpressed cells comparing only treatment of Aβ. (+, present; -, absent. Bar depict mean ± S.D. of at least three independent experiments. " * " represent a statistically significant difference from control group or the same group, p<0.05. " § " represent a statistically significant difference from different group treat with β , p<0.05)

5. The expression of Nanog were mediated by mir-302s overexpressed cell though maintaining Akt/ GSK3β signaling

The overexpression of mir-302s regulated Akt/ GSK3β signaling, which provided a preventive effect against Aβ-induced neurotoxicity through Nanog expression. (A) After treatment of Aβ (2.5 μM) incubated 24 h, the Nanog protein expression regulated the pSer 473 Akt protein expression by western blotting. (B) When we analyzed knock-down of Nanog in mir-302s overexpressed cells by transient transfection, the Nanog expressions were decreased. (C) We also treated Aβ (2.5 μM) into transiently knock-down of Nanog cells for 24 h, the knock-down of Nanog regulated the expression of p-Akt and p-GSK3β comparing with mir-302s overexpressed cell only treating Aβ. (D) The knock-down of Nanog also affected the p-IRS-1, IRS-1 and tyrosine expression after Aβ-treating comparing with mir-302s overexpressed cell only treating Aβ. (+, present; -, absent. Bar depict mean ± S.D. of at least three independent experiments. " * " represent a statistically significant difference from control group or the same group, p<0.05. " § " represent a statistically significant difference from different group treat with β , p<0.05)

6. The expression of Nanog was detected in PBMC of AD patient

Nanog expression levels were decreased in AD patients' PBMC. To detect mRNA levels of Nanog expression, we collected AD patients' and normal age-match control's blood samples, and extracted mRNA for PBMC. (A) The mRNA levels of Nanog was detect by real-time PCR, AD patients was lower expression of Nanog mRNA comparing with control. (Bar depict mean ± S.D. of at least three independent experiments. " * " represent a statistically significant difference from control group, p<0.05)

重要性及影響性

根據我們的實驗結果獲致以下的結論：

1. 累積於腦部的 Aβ 其誘發之細胞毒性其機制可能是經由阻礙神經細胞的 insulin signaling 而導致神經退化，本研究易結果證實透過減緩 brain insulin resistance 可有效抑制 Aβ 所誘發之神經毒性。

2. 我們的實驗釐清了 insulin signaling 與 Aβ 神經毒性之間的關係，特別是首先發現 Nanog 這個幹細胞相關蛋白在此機制中所扮演的角色，並證實可透過調控 insulin signaling 來影響細胞內 Nanog 表現，顯示將其應用於生物標記作為輔

助 AD 臨床診斷的可能性。

3. 在 T2DM 治療上，metformin 與 TZD 是最被廣泛使用的第一線藥物，然而其對 AD 治療效果的分子機轉則有待實證。本研究結果證實透過改善 insulin resistance 及活化 AMPK 這兩種方式，都能幫助神經細胞對抗 Aβ 傷害，此結論將可提供部分 anti-diabetic 藥物在未來用於合併治療 T2DM 及 AD 的概念。

4. 雖然幹細胞療法是治療人類神經病變的新遠景之一，但在胚胎幹細胞在使用及取得方面卻有其難以逾越的法律與倫理上的問題。而我們首先嘗試利用 iPS 方式，測試其應用在對抗 Aβ 神經毒性的可能潛力，預期這種 neuronal iPS 將可發揮幹細胞的生物特性，配合受損神經組織微環境的變化，動態地增進各種神經保護因子的旁分泌(paracrine)作用，同時並抑制膠細胞的過度活化及免疫反應，因此得以讓已受 Aβ 損傷的神經元有機會修復重生。

5. 已知 Aβ 具有抑制 autophagy 的能力，而 AMPK、Sirt1、PGC1α 及 autophagy 都是跟老化高度相關的蛋白，本研究也發現 insulin signaling 與這些老化相關蛋白之間的互動關係，今後並可能因此開發出全新思維的 AD 治療策略。

附錄一：以下為計畫執行期間根據本計畫研究結果/經費補助發表之著作：

期刊論文

1. Liao CL, Chen CM, Chang YZ, Liu GY, Hung HC, Hsieh TY, <u>Lin CL</u>[*]. Pine (*Pinus morrisonicola Hayata*) Needle Extracts Sensitize GBM8901 Human Glioblastoma Cells to Temozolomide by Downregulating Autophagy and O^6-Methylguanine-DNA Methyltransferase Expression. *J. Agri. Food Chem.* *62:10458-10467, 2014.* (SCI) (IF=2.912, Ranking=2/56; **3.6%** of AGRICULTURE, MULTIDISCIPLINARY）

2. Chen CM, Syu JP, Way TD, Huang LJ, Kuo SC, Lin CT, <u>Lin CL</u>[*]. BC3EE2,9B, a synthetic carbazole derivative, upregulates autophagy and synergistically sensitizes human GBM 8901 glioblastoma cells to temozolomide. *Int. J. Mol. Med.* *36:1244-1252, 2015* (SCI) (IF=2.088, Ranking=70/123; **56.9%** of MEDICINE, RESEARCH & EXPERIMENTAL）

3. Tung WF, Chen WJ, Hung HC, Liu GY, Tung JN, Huang CC, <u>Lin CL</u>[*]. 4-Phenylbutyric acid (4-PBA) and lithium cooperatively attenuate cell death during oxygen-glucose deprivation (OGD) and reoxygenation. *Cell. Mol. Neurobiol.* *35:849-859, 2015.* (SCI) (IF=2.506, Ranking=152/252; **60.3%** of NEUROSCIENCES)

4. Li HH, Lu FJ, Hung HC, Liu GY, Lai TJ, <u>Lin CL</u>[*]. Humic acid increases amyloid β-induced cytotoxicity by induction of ER stress in human SK-N-MC neuronal cells. *Int. J. Mol. Sci.* *16: 10426-10442, 2015.* (SCI) (IF=2.862, Ranking=45/157; **28.7%** of CHEMISTRY, MULTIDISCIPLINARY）

5. Kornelius E[#], <u>Lin CL</u>[#], Chang HH, Li HH, Huang WN, Yang YS, Lu YL, Peng CH, Huang CN. DPP-4 inhibitor linagliptin attenuates Aβ-induced cytotoxicity through activation of AMPK in neuronal cells. *CNS Neurosci. Ther.* *21:549-57, 2015.* (SCI) (IF=3.931, Ranking=43/254; **16.9%** of PHARMACOLOGY & PHARMACY） ([#]the first two authors contributed equally)

6. <u>Lin CL</u>, Huang WN, Li HH, Huang CN, Hsieh S, Lai C, Lu FJ. Hydrogen-rich water attenuates amyloid β-induced cytotoxicity through upregulation of Sirt1-FoxO3a by stimulation of AMP-activated protein kinase in SK-N-MC cells. *Chem. Biol. Interact.* *240:12-21, 2015.* (SCI) (IF=2.577, Ranking=110/254; **43.3%** of PHARMACOLOGY & PHARMACY）

7. <u>Lin CL</u>[*]. Attenuation of endoplasmic reticulum stress as a treatment strategy against ischemia/reperfusion injury. (Invited Review) *Neural. Regen. Res. (In press)* (SCI) (IF=0.220, Ranking=247/252; **98.0%** of NEUROSCIENCES)

8. <u>Lin CL</u>, Cheng YS, Li HH, Chiu PY, Chang YT, Ho YJ, Lai TJ. Amyloid-β suppresses AMP-activated protein kinase (AMPK) signaling and contributes to α-synuclein-induced cytotoxicity. *Exp. Neurol. (In press)* (SCI) (IF=4.696, Ranking=47/252; **18.7%** of NEUROSCIENCES）

研討會論文

1. Tung WF, Chen WJ, Lai TJ, Lee HH, Tung JN, <u>Lin CL</u>. 4-Phenylbutyrate combined with lithium salt attenuates hypoxia/reoxygenation-induced neuronal endoplasmic reticulum stress and apoptosis. *26th Joint Annual Conference of Biomedical Science. P433, Taipei, Taiwan, Mar 2011.*

2. Luo PQ, Yang JY, Liu GY, <u>Lin CL</u>. Assessments of a novel alumina nanomaterial on microglial immune modulation. *26th Joint Annual Conference of Biomedical Science. P434, Taipei, Taiwan, Mar 2011.*

3. <u>Lin CL</u>, Luo CY. Simvastatin promotes neuronal survival following amyloid β-induced neurotoxicity through AMP-activated protein kinase activation. *Alzheimer's Association International Conference on Alzheimer's Disease. P2-243, Paris, France, July 2011.*

4. <u>Lin CL</u>, Huang WN, Lee HH, Liu GY, Hung HC. Amyloid β interferes with assembly of tubulin by an antizyme dependent pathway in neuronal cells. *The 8th FENS Forum of Neuroscience. C102, Barcelona, Spain, July 2012.*

5. <u>Lin CL</u>, Li HH, Cheng YS, Lai TJ. Amyloid β contributes to the neurotoxic effects of α-synuclein aggregates in neuronal cells. *International Congress of Toxicology 2013. P1-072, Seoul, Korea, June 2013.*

6. Li HH, Lai TJ, Lin SL, Lu FJ, Huang WN, Cheng YS, Chang HH, <u>Lin CL</u>. The insulin signaling cascade modulated Aβ induced cell death by the generation of human ES-like miR302-induced pluripotent-stem-cell-like State. *28th Joint Annual Conference of Biomedical Science. P228, Taipei, Taiwan, Mar 2013.*

7. Huang WN, <u>Lin CL</u>, Li HH, Chang HH, Cheng YS, Lu FJ. Hydrogen-rich water against Aβ-induced cell death through AKT/SIRT1/FOXO3a modulating in human SK-N-MC cells. *28th Joint Annual Conference of Biomedical Science. P719, Taipei, Taiwan, Mar 2013.*

8. Li HH, Lai TJ, Lin SL, Lu FJ, Huang WN, Cheng YS, Chang HH, <u>Lin CL</u>. The insulin signaling cascade modulated Aβ induced oxidative stress by the generation of human ES-like mir302-induced pluripotent-stem-cell-like state. *6thBiennial Meeting of Society for Free Radical Research-Asia. P10. Taoyuan, Taiwan, October 2013.*

9. Huang WN, <u>Lin CL</u>, Li HH, Chang HH, Cheng YS, Lu FJ. Hydrogen-rich water against Aβ-induced apoptosis in SK-N-MC cells through regulation of AMPK/SIRT1/FOXO3A. *6thBiennial Meeting of Society for Free Radical Research-Asia. P11. Taoyuan, Taiwan, October 2013.*

10. Cheng YS, Chiu PY, Li HH, Chang HH, <u>Lin CL</u>, Lai TJ. Investigating the molecular mechanisms of amyloid β mediates α-synuclein-induced neurotoxicity.

29th Joint Annual Conference of Biomedical Science. P124, Taipei, Taiwan, Mar 2014.

11. Cheng YS, Li HH, Chiu PY, Chang HH, <u>Lin CL</u>, Lai TJ. α-Synuclein, amyloid β and autophagy inhibition in dementia with Lewy bodies. *9th Annual Conference of Taiwanese Society of Geriatric Psychiatry. Taichung, Taiwan, Mar 2014.*

12. Li HH, Lai TJ, <u>Lin CL</u>. Generation of Alzheimer's disease-associated amyloid precursor protein C99 conditional expression model in a human neuronal cell line SK-N-MC. *The 3rd International Congress on Natural Sciences and Engineering. P731. Kyoto, Japan, May 2014.*

13. <u>Lin CL</u>, Huang CN, Chang HH, Li HH. Dipeptidyl peptidase-4 inhibitor attenuates amyloid β-induced neuronal insulin signaling blockade. *The 8th FENS Forum of Neuroscience. C025, Milan, Italy, July 2014.*

14. Lai TJ, Chang YT, Tsai SK, Kim HG, Tsai YJ, Li HH, Huang WN, Ho, YJ, <u>Lin CL</u>. Molecular interplay between Aβ and mTOR signaling in α-synuclein-induced cognitive decline. *10th Annual Conference of Taiwanese Society of Geriatric Psychiatry. G28, Taichung, Taiwan, Mar 2015.*

15. Tsai YJ, <u>Lin CL</u>, Huang CN, Li HH, Huang WN, Tsai SK, Chang YT, Kim HG, Lu FJ. Hydrogen-rich water protects free acid-induced hepatic insulin resistance. *30th Joint Annual Conference of Biomedical Science. P733, Taipei, Taiwan, Mar 2015.*

16. <u>Lin CL</u>, Kim HG, Li HH, Huang WN, Huang CN. Induction of Nanog displays protective effects against amyloid β (Aβ)-induced cytotoxicity. *40th FEBS Congress. P22-011, Berlin, Germany, July 2015.*

附錄二：根據本計畫研究結果/經費補助著作(minor revision/尚未發表)之原稿

1. Li HH, Lin SL, Huang CN, Lu FJ, Chiu PY, Huang WN, Lai TJ, <u>Lin CL</u>[*]. miR-302 Attenuates Aβ-induced Neurotoxicity through Activation of Akt Signaling. *J. Alzheimers Dis.* **(minor revision)** (SCI) (IF=4.151, Ranking=58/252; **23.0%** of NEUROSCIENCES)

2. Wei J. CC, Huang HC, Chen WJ, Huang CN, Peng CH, <u>Lin CL</u>[*]. Epigallocatechin gallate attenuates amyloid β-induced inflammation and neurotoxicity in EOC 13.31 microglia. *Eur. J. Pharm.* **(minor revision)** (SCI) (IF=2.532, Ranking=133/254; **52.2%** of PHARMACOLOGY & PHARMACY)

miR-302 Attenuates Aβ-induced Neurotoxicity through Activation of Akt Signaling

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Abstract

Deficiency of insulin signaling has been linked to diabetes and ageing-related neurodegenerative diseases such as Alzheimer disease (AD). In this regard, brains exhibit defective insulin receptor substrate-1 (IRS-1) and hence result in alteration of insulin signaling in progression of AD, the most common cause of dementia. Consequently, dysregulation of insulin signaling plays an important role in amyloid β (Aβ)-induced neurotoxicity. As the derivation of induced pluripotent stem cells (iPSC) involves cell reprogramming, it may provide a means for regaining the control of ageing-associated dysfunction and neurodegeneration via affecting insulin-related signaling. To this, we found that an embryonic stem cell (ESC)-specific microRNA, miR-302, silences phosphatase and tensin homolog (PTEN) to activate Akt signaling, which subsequently stimulates nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) elevation and hence inhibits Aβ-induced neurotoxicity. miR-302 is predominantly expressed in iPSCs and is known to regulate several important biological processes of anti-oxidative stress, anti-apoptosis and anti-aging through activating Akt signaling. In addition, we also found that miR-302-mediated Akt signaling further stimulates Nanog expression to suppress Aβ-induced p-Ser307 IRS-1 expression and thus enhances tyrosine phosphorylation and p-Ser 473-Akt/ p-Ser 9-GSK3β formation. Furthermore, our *in vivo* studies revealed that the mRNA

2

expression levels of both *Nanog* and miR-302-encoding *LARP7* genes were significantly reduced in AD patients' blood cells, providing a novel diagnosis marker for AD. Taken together, our findings demonstrated that miR-302 is able to inhibit Aβ-induced cytotoxicity via activating Akt signaling to upregulate Nrf2 and Nanog expressions, leading to a marked restoration of insulin signaling in AD neurons.

Keywords: Alzheimer disease; insulin signaling; amyloid β; miR-302; Nanog; Phosphatase and tensin homolog

INTRODUCTION

Insulin, a hormone made and secreted by the pancreas, has great potent effects on our brains [1]. Insulin resistance represents a loss or reduction in its normal functionality on target tissues and hence affects our cognitive and memory functions, ultimately leading to the onset of Alzheimer disease (AD) [2]. Insulin resistance has been linked to several previously identified risk factors that accelerate the cognitive dysfunction and ageing process, including diabetes, obesity, hypertension, hyperlipidemia, and metabolic syndrome [3]. Particularly, brains exhibit defective insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) show alteration or aberrant activation of insulin signaling in progression of AD, the most common cause of dementia [4]. These findings suggest that neuronal insulin signaling becomes dysfunction in the AD brains similar to the dementia symptoms of Type 2 diabetes. The pathogenesis of AD is initially triggered by the presence of extracellular amyloid-β (Aβ) peptides, which impair mitochondrial membrane potential (MMP) and contribute to an increase in the accumulation of intracellular reactive oxygen species (ROS), ultimately resulting in neuronal cell death [5, 6]. It has been well established that Aβ deposition may play a pathogenic role in age-associated AD pathogenesis [7]. In addition, our previous studies have indicated that Aβ induces p-Ser307 IRS-1 expression and inhibits IRS-1 tyrosine phosphorylation and its

downstream target protein kinase B (PKB, also known as Akt) [8]. Subsequently, Aβ

further suppresses Ser9 phosphorylation of glycogen synthase kinase 3β (GSK3β),

which is one of the enzymes responsible for causing tau hyperphosphorylation and

neurotoxicity [9]. These findings all indicate that insulin signaling plays an important

regulatory role in Aβ-induced neurotoxicity.

MicroRNAs (miRNAs) are short single-stranded noncoding RNAs (approx. 20-

to 25-nucleotide long) representing a class of small regulatory RNAs. miRNAs inhibit

targeted gene expression by suppressing mRNA translation and thus they play an

regulatory role in a wide range of cellular processes [10]. Emerging evidence has

shown that aberrant miRNA expression is involved in the development or progression

of neurodegenerative disorders including AD [10-12]. Indeed, alterations in miRNA

expression have been reported to potentially modulate the levels of toxic Aβ species

and subsequently induce neuronal death [10]. However, the function of these miRNAs

involved in Aβ-induced neurotoxicity as well as neurodegenerative diseases remains

unclear. As recent studies also showed that embryonic stem cell (ESC)-specific

miRNAs play a pivotal role in somatic cell reprogramming [13, 14], of which the

reprogramming process may help to reset diseased gene profiles into a relatively

normal state. To this, regenerative medicine using such a reprogramming mechanism

holds a great promise in developing therapies for treating degenerative diseases [13,

14].

Previously, we have shown that an ESC-specific miRNA, the miR-302 family, was responsible for regulating the pluripotency of human ESC and induced pluripotent stem cells (iPSCs) [15-17]. The genomic sequence encoding for miR-302 is located in the intron of the La ribonucleoprotein domain family member 7 (LARP7) gene on human chromosome 4, a conserved region frequently associated with longevity [18]. Functional studies also identified that miR-302 governs self-renewal by dually regulating oxidative stress [19], and apoptosis [15, 20]. It suggested that miR-302 may prevent apoptosis via downregulating apoptotic-associated genes and upregulating anti-apoptotic genes [19]. In addition, miR-302 also inhibits oxidant-induced cell death, likely by targeting oxidative-associated genes. Hence, miR-302 is capable of regulating self-renewal and pluripotency simultaneously through modulating oxidative stress, and apoptosis. Yet, it is still unclear whether miR-302 plays a role in preventing Aβ-induced neurotoxicity. To this, we herein investigated whether miR-302 exhibits a neuroprotective effect against Aβ-induced neurotoxicity in human neuronal cells.

Maintaining cell survival is dependent on external factors such as growth factors, the lack of which may initiate cellular apoptosis. The Akt signaling pathway has been established as a major downstream effector of growth factor-mediated cell survival,

and has been shown to inhibit apoptosis [21]. Akt promotes cell survival by inactivating certain pro-apoptotic mediators such as Bid, a pro-apoptotic member of the Bcl-2 family involved in the induction of death receptor-mediated apoptosis [22]. In addition, Akt signaling is able to reduce oxidative stress, which in turn may play a role in activating nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) axis [23]. Activation of Nrf2/HO-1 antioxidant pathway leads to the prevention of Aβ-induced neurotoxicity [24]. Both oxidative stress and apoptosis are the underlying mechanisms involved in Akt signaling pathway in neurodegeneration and mitochondrial dysfunction. Taken together, these observations strongly suggest that the Akt signaling pathway plays an important role in cell survival, anti-oxidative stress, neuroprotection, and pluripotency. Interestingly, miR-302 has been reported to mediate Akt activation through downregulating phosphatase and tensin homolog (PTEN) in maintaining the pluripotent status of ESCs [25]. PTEN is a key negative regulator of phosphoinositide 3-kinase (PI3K)/Akt signaling. PI3K/Akt pathway is also a common mediator of pluripotency and self-renewal in ESCs. Moreover, Akt signaling regulates the pluripotency-associated gene Nanog to maintain stem cell self-renewal, pluripotency [26] and anti-ageing [27]. In the present study, we postulate that neuronal insulin resistance represents one of the mechanisms underlying Aβ-mediated neurotoxicity, and miR-302 can protect neuronal cells by restoring

impaired insulin signaling via the prevention of Aβ-induced neurotoxicity.

MATERIALS AND METHODS

Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), dihydroethidium (DHE), and JC-1 were purchased from Sigma (Munchen, Germany). Amyloid β (Aβ) 1-42 was acquired from AnaSpec Inc. (San Jose, CA, USA), and solutions were prepared according to our previous report [6]. Antibodies against Akt, p-Akt, GSK3β, p-GSK3β, IRS-1, Nrf2, HO-1, tBid, Caspase 3, and poly (ADP-ribose) polymerase (PARP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and p-tyrosine, p-Tau, and Tau were obtained from Merck Millipore (Darmstadt, Germany). On the other hand, β-actin antibody was obtained from Novus Biologicals. (Littleton, CO, USA) and p-IRS-1, Nanog and PTEN antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Lastly, the antibody against SOD-1 was purchased from Genetex (San Antonio, TX, USA).

Cell culture

Human neuroblastoma SK-N-MC cells were obtained from the American Type

Culture Collection (ATCC, Bethesda, MD, USA). Cells were maintained in Minimal

Eagle's medium (MEM, Gibco), supplemented with 10% fetal bovine serum, 100

units/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine at 37 ℃, 5%

CO_2. For inducing miR-302 expression, a pLVX-miR-302 vector was modified from

Clontech's pLVX-AcGFP plasmid as previously reported [28]. Then, the SK-N-MC

cells were transfected with the pLVX-mir302 vector to form miR-302-overexpressed

cells, using a lipofectamine 2000 reagent (Invitrogen) following the manufacturer's

instructions. The miR-302-overexpressed cells were identified by the presence of a

co-expressed AcGFP green fluorescent protein. For silencing Nanog expression,

another shRNA gene silencer vector directed against human Nanog mRNAs, called

shRNA-Nanog, was obtained from Academia Sinica in Taiwan. In some experiments,

we further transfected the shRNA-Nanog vector into the miR-302-overexpressed cells

with the lipofectamine 2000 reagent.

Cell viability assay

Cells were seeded in 24-well plates overnight and then treated as indicated. After

24 h, the tetrazolium salt MTT was added to the medium following the manufacturer's

instructions. Only viable cells could metabolize MTT into a purple formazan product,

of which the color density (OD) was further quantified by a Bio-Rad

spectrophotometer at 550 nm. Cell viability was determined by the percentage of OD

from treated cells or transfected cells divided by OD from control cells.

Nucleus morphology

Cells were cultivated on coated slides at 60% confluency and then treated with

drugs for 24 h. Thereafter, changes in cell nucleus morphology, in particular

characteristics of apoptosis, were examined, using a fluorescence microscope. The

cells were fixed in 4% paraformaldehyde after 24 h of treatment with the indicated

compounds, permeabilized in ice-cold methanol, incubated with 1 ng/mL of DAPI

stain for 15 min at room temperature, and then observed under a fluorescence

microscope (DP80/BX53, Olympus). Apoptotic cells were quantified by counting four

random fields per condition of treatment.

Western blot analysis

Cells were harvested and homogenized with a protein extraction lysis buffer

containing 50 mM Tris-HCl, pH 8.0; 5 mM ethylenediaminetetraacetic acid (EDTA);

150mM sodium chloride (NaCl); 0.5% Nonidet P-40; 0.5 mM dithiothreitol (DDT); 1

mM phenylmethylsulfonyl fluoride (PMSF); 0.15 units/ml aprotinin; 5 μg/ml

Leupeptin; 1 μg/ml pepstatin; and 1mM sodium fluoride (NaF), and then centrifuged

at 12,000 g for 30 min at 4℃. The supernatant cell lysate was used for

immunoblotting analysis. Equal amounts (50 μg) of total proteins from the cell lysate were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore), and then probed with a primary antibody followed by another secondary antibody conjugated with horseradish peroxidase. Primary antibodies were used at a dilution of 1:1000 in 0.1% Tween-20 and secondary antibodies were used at 1:5000 dilutions. The immunocomplexes were visualized using enhanced chemiluminescence kits (Millipore). The relative expression levels of proteins were quantified densitometrically using the QuantityOne software (BioRad), and then further calculated according to the reference bands of β-actin. Each blot represents data from at least three independent tests. Signals were quantified relative to the level of control cellls.

Analysis of mitochondrial membrane potential (MMP)

MMP was investigated using a vital mitochondrial cationic dye JC-1, which accumulates in mitochondria in a potential-dependent matter. Cells were treated with 1 μM of JC-1 in fresh medium and incubated at 37℃ for 30 min. Cell morphology was then observed and photographed using an inverted fluorescence microscope (DP72/CKX41, Olympus). In normal cells, JC-1 remained as red fluorescent aggregations, whereas during the induction of apoptosis the mitochondrial potential

collapsed and hence JC-1 formed monomers producing green fluorescence. MMP was quantified by fluorescent intensity using Image J software (NIH, Bethesda, MD). The levels of relative fluorescent intensities were shown in percentage of measured intensity in miR-302-transfected cells compared to that in non-transfected control cells.

Detection of ROS by dihydroethidium (DHE) staining

DHE is a fluorogenic reagent used for detecting intracellular superoxide radical anion [29]. Cells were treated in fresh medium containing 10 μM DHE and incubated for 30 min in the dark at room temperature. After 30-min incubation, the staining medium was discarded and the cells were washed twice with PBS and then observed and photographed under an inverted fluorescence microscope (DP72/CKX41). ROS levels were determined by oxidized DHE fluorescence intensity using Image J software (NIH, Bethesda, MD). The levels of fluorescent intensities were quantified relative to that of control cells.

Study population and blood samples

Blood sampling from AD patients (n=7) and age-matched healthy individuals (n=6) was performed according to standardized procedures approved by the

Institutional Review Board (IRB) of Chung Shan Medical University Hospital (CSMUH No: CS 13233) (Table 1). Clinical AD diagnosis was determined by the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) criteria and completed with a Mini-Mental State Examination (MMSE) and cognitive abilities screening instrument (CASI) test. MMSE scores were used as a rough measurement of cognitive function. CASI scores ranged from 1 to 100 were used for quantitative assessment on attention, concentration, orientation, short-term memory, long-term memory, language abilities, visual construction, list-generating fluency, abstraction, and judgment. A detailed overview of AD patients (n = 7, mean age 80.0 ± 4.9 years, range 74-86 years) and age-matched healthy individuals (n = 6, mean age 80.0 ± 5.9 years, range 72-86 years) was summarized in Table 1. A number of AD patients (n=7, Female/Male = 4/3) had moderate dementia under MMSE (mean scale = 19.3 ± 2.6, range 16-23) and CASI (mean scale = 65.1 ± 10.3, range 49-79) measurement scales, showing most differences between AD patients and age-matched healthy controls (n = 6, Female/Male = 3/3) (Table. 1). Age-matched healthy individuals were recruited by local advertisement at the Aging Research Unit, Chung Shan Medical University, Taichung, Taiwan. Neither cognitive impairment nor any dementia disorder was detected in all tested healthy individuals. Both AD patients and age-matched healthy individuals were volunteers with written informed consents obtained from the patients

or their legal guarding relatives, according to the Declaration of Helsinki and the

IRB-approved protocols. Approximately 20 mL of venous peripheral blood

mononucleated cells (PBMCs) were obtained from each tested subject and then total

RNAs were isolated from each blood sample with a Oiagen RNeasy Kit (Qiagen,

Germantown, MD, USA) and further used for spectrophotometric quantification

following the manufacturer's instructions.

Reverse transcription (RT) and quantitative PCR (qPCR)

Total RNAs were extracted from patients' PBMCs and SK-N-MC cells, respectively,

using a Qiagen RNeasy Kit (Qiagen) and further quantified spectrophotometrically.

RT-qPCR was carried out using 1 μg of total RNAs and following the protocols of an

ABI High-Capacity cDNA Archive Kit (ABI). Then, we diluted the resulting cDNA

into ten folds and used only 5 μl of the diluted cDNA in each of triplicate qPCRs run

on a Applied Biosystems 7300 Real Time PCR System with Maxima SYBR Green

qPCR Master Mix (2X), ROX solution provided (Thermo), according to the

manufacturer's instructions. Primers were listed in Supplemental Table 1. Levels of

relative mRNA or miRNA expression were acquired withthe SDS software version

1.2.3 (Sequence Detection Systems 1.2.3-7300 Real Time PCR System, Applied

Biosystems) and then further standardized (**normalized?**) with the level of

housekeeping GAPDH expression in the same sample. Each bar represents data from at least three independent tests. Samples from untreated cells or normal healthy individuals were used as a control.

Indirect detection of endogenous miR-302

The genomic sequence encoding for the miR-302 cluster is located in the intron of the *LARP7* gene on human chromosome 4 [18]. The endogenous miR-302 familial cluster is produced by RNA splicing of the intron between exons 8 and 9 of *LARP7*. After intron splicing, the two exons were joined together to for mature mRNAs, of which the joined sites was targeted by a pair of specially designed primers for detecting the expression and splicing of the miR-302 cluster RNAs derived from the *LARP7* gene transcripts (Supplemental Fig. 1). The primers were named as LARP7-forward and LARP7-reverse in the list of Supplemental Table 1.

Statistical analysis

Each experiment was repeated for three times (n = 3). All data were presented as means ± standard error of mean (S.E.M). For cell viability tests, the average population number of control cells was set as a 100% standard for comparing the survival rates of other tested cells. For Western blotting and RT-qPCR, the measured

amounts of protein or mRNA expression were first normalized with the expression level of housekeeping ß-actin protein or mRNA, respectively. Then, the normalized protein and mRNA levels from control cells or normal healthy individuals were used as a 100% standard for comparing the relative expression levels of the same protein or mRNA in tested groups. Statistical significance of differences between compared groups was determined by one-way analysis of variance (ANOVA) following Dunnett's *post-hoc test* for multiple comparisons with a SPSS statistical software (SPSS, Inc., Chicago, IL, USA) as well as the two-tailed Student's t-test. A probability value of <0.05 or < 0.01 was taken to indicate statistical significance and hence the significant levels were set at * $p < 0.05$ or ** $p < 0.01$, respectively, depending on individual experiments.

RESULTS

miR-302 protects SK-N-MC cells against Aβ-induced apoptosis

Recent studies have demonstrated the crucial functions of miR-302 in regulating oxidative stress-induced apoptosis [20]. To address whether miR-302 exerts any protective effect on neuronal cells against Aβ-induced apoptosis, we transfected human neuronal SK-N-MC cells with a cytomegalovirus (CMV)-promoter-driven miR-302 expression vector as previously reported [17], and then exposed to Aβ (2.5

16

µM) for 24 hours. After that, the transfected miR-302-overexpressed cells were identified by the presence of a co-expressed AcGFP green fluorescent protein under an inverted fluorescent microscope (Fig. 1A) and the expression of miR-302 was further confirmed by RT-qPCR (n = 3, p<0.01, Fig. 1B) and miRNA microarray analysis (Supplementary Data), showing successful transcription of the whole miR-302 familial cluster (*i.e.* miR-302a, b, c, and d). Notably, Fig. 1C further demonstrated that Aβ treatment triggered massive cell death in control cell groups, whereas miR-302-overexpressed cells showed marked attenuation of such Aβ-induced cell death (n = 3, p<0.01). To determine which kind of cell death induced by Aβ, we further examined the nuclei fragmentation by DAPI staining. As shown in Fig. 1D, Aβ treatment disrupted nucleus margin and significantly increased the apoptotic cell population in the control groups compared to those of miR-302-overexpressed cells (n = 3, p<0.01). In addition, Fig. 1E revealed that Aβ treatment markedly increased the cleavage formation of both caspase 3 and PARP in control cells but not in miR-302-overexpressed cells (n = 3, p<0.01), further confirming this point. Taken together, our data strongly suggest that miR-302 plays a protective role in preventing Aβ-induced cell apoptosis.

Activation of Akt signaling is involved in miR-302-mediated neuroprotection

We have previously reported that restoration of insulin sensitivity in neurons leads to Akt activation and so as to inhibit Aβ-induced apoptosis [8]. To determine whether miR-302 expression can restore neuronal insulin sensitivity and prevent Aβ-induced neurotoxicity, we used Western blot analyses to measure the expression levels of major insulin signaling-related proteins, such as pSer307-IRS-1, tyrosine phosphorylation of IRS-1, and their downstream target pSer473-Akt. As shown in Fig 2A, Aβ treatment in control cells significantly increased p-307 IRS-1 serine phosphorylation (n = 3, p<0.05) while decreasing IRS-1 tyrosine phosphorylation (n = 3, p<0.01), both of which are considered as hallmarks of insulin resistance; yet, in miR-302-overexpressed cells this Aβ-induced insulin resistance was markedly attenuated (p<0.05). Moreover, Aβ treatment also led to a significant decrease of p-Ser 473-Akt in control groups but not in miR-302-overexpressed cells (n = 3, p<0.01) (Fig. 2A). To further elucidate the protective role of PI3K/Akt signaling in miR-302-overexpressed cells, we applied a PI3K inhibitor - LY294002. Fig. 2B revealed that co-treatment of Aβ (2.5 μM) and LY294002 (20 μM) could disrupt miR-302-mediated Akt signaling (p<0.01) and thus resulted in a marked reduction of the viable cell population, as determined by MTT assay (p<0.01, Fig. 2C). All these findings suggest that miR-302 prevents Aβ-induced neurotoxicity and neuronal death via activating PI3K/Akt signaling. Alternatively, Aβ-impaired insulin signaling may

also lead to an increase of GSK3β activity as well as tau hyperphosphorylation, a relevant step in AD pathogenesis [30]. To this, we found that miR-302 expression could stimulate Akt signaling to slightly increase p-Ser 9-GSK3β levels and hence may provide a mild inhibitory effect on tau hyperphosphorylation (n = 3, p<0.05) (Fig. 2D). As a result, Fig. 2D also showed that co-treatment of Aβ and LY294002 totally abolished the inhibitory effect of miR-302 on p-Ser 9-GSK3β expression and tau phosphorylation in control cells compared to those of miR-302-overexpressed cells (n = 3, p<0.05). Taken together, our data demonstrate that miR-302 may exert its protective effects mainly through activating and/or restoring the Akt/GSK3β signaling pathway.

miR-302 attenuates Aβ-induced oxidative stress through Akt-upregulated Nrf2/HO-1

Emerging evidence suggested that Aβ is able to generate free radicals and oxidative damages [6]; yet, activation of Akt signaling may inhibit such Aβ-induced oxidative stress and apoptosis [31]. To determine whether miR-302-mediated Akt activation can prevent Aβ-induced intracellular ROS accumulation, we performed a fluorometric assay to measure the concentration of hydrogen peroxide accumulated in the cells. As shown in Fig. 3A, Aβ treatment stimulated a significant elevation of intracellular superoxide radical anions in control groups but not in

miR-302-overexpressed cells (n = 3, p<0.01). Co-treatment of Aβ (2.5 μM) and

insulin (1 μM) could restore the normal levels of intracellular superoxide radical

anions in control groups (p<0.05), indicating that miR-302-mediated Akt activation

did inhibit Aβ-induced ROS. Furthermore, recent studies also indicated that Nrf2, a

redox-sensitive transcription factor, can confer protection against ROS damage by

upregulating antioxidant-response elements, such as HO-1 [23, 32]. Since PI3K/Akt

signaling has been reported to elevate HO-1 expression and Nrf2-dependent

transcription [24], we further elucidate this possible anti-oxidant effect of miR-302 by

Western blot assays. As a result, Fig. 3B revealed that Aβ treatment reduced both Nrf2

and HO-1 expressions in control groups but not in miR-302-overexpressed cells (n =

3, p<0.05). To confirm the source of this effect, further treatment of LY294002 (20

μM) with Aβ (2.5 μM) also decreased Nrf2 expression in miR-302-overexpressed

cells (n = 3, p<0.05) (Fig. 3C), indicating that miR-302 regulates Nrf2 expression via

the PI3K/Akt signaling pathway. Moreover, activation of Akt signaling significantly

restored the Nrf2 expression after co-treatment of Aβ (2.5 μM) and insulin (1 μM) in

control groups (p<0.05) (Fig. 3C), further suggesting that miR-302-mediated Akt

activation can prevent Aβ-induced ROS accumulation through the upregulation of

Nrf2 and HO-1.

To investigate the miR-302 effect on Aβ-mediated mitochondria dysfunction and

apoptosis, we examined MMP with JC-1 staining assays and the expression of apoptotic-associated marker truncated Bid (tBid) and anti-apoptotic-associated marker Bcl-2 with Western blotting assays. As shown in Fig. 3D, control cells displayed a significant deficiency of mitochondrial membrane depolarization in response to Aβ treatment (n = 3, p<0.05), which was however not found in miR-302-overexpressing cells, as indicated by the concurrent loss of cytoplasmic red J-aggregate fluorescence and elevation of diffused green fluorescence. Yet, this miR-302-mediated protective effect on MMP integrity could be totally abolished by co-treatment of Aβ (2.5 μM) and LY294002 (20 μM) for 24 hours (n = 3, p<0.05), indicating the involvement of Akt/PI3K signaling. In addition, Aβ treatment resulted in a marked increase of tBid expression (p<0.01) and decrease of Bcl-2 (p<0.05) in control groups, but not in miR-302-overexpressed cells (Fig. 3E). All these findings clearly suggest that miR-302-mediated Akt activation can inhibit Aβ-induced oxidative stress, mitochondria dysfunction and apoptosis via upregulating Nrf2 activities.

miR-302 regulates Akt signaling by targeting PTEN and inducing Nanog expression

After having determined the important role of miR-302 in activating Akt signaling to prevent Aβ-induced neurotoxicity, we further investigate the molecular mechanism underlying such miR-302-mediated Akt activation. Recent studies have

indicated that miR-302 promotes pluripotency through Akt signaling by targeting

PTEN [25]. To search the miR-302 target site in PTEN, we performed screening

analyses using a prediction program, TargetScan (http://www.targetscan.org/), and

identified a specific miR-302 binding site located in the 3'UTR of human PTEN gene

(Fig. 4A). As our Western blotting data have shown a significantly decrease of PTEN

expression in miR-302-overexpressed cells (n = 3, p<0.05) (Fig. 4B), it suggests that

miR-302 may target this 3'UTR binding site to suppress PTEN expression. Also,

since knockdown of PTEN can increase the pluripotency-associated gene Nanog

expression [26], which is further mediated by PI3K/Akt signaling in ESCs [25], we

herein examined the miR-302 effects on PTEN, pSer473 Akt, and Nanog expressions

with Western blot assays. As a result, Fig. 4C showed a marked elevation of Nanog

expression only detected in miR-302-overexpressed cells (n = 3, p<0.05), while Aβ

treatment (2.5 μM for 24 hours) stimulated a significant increase of PTEN as well as

decreases of pSer473 Akt and Nanog expressions in control groups but not in

miR-302-overexpressed cells (n = 3, p<0.05) (Fig. 4D). Interestingly, further studies

revealed that blocking Akt signaling with LY294002 (20 μM for 24 hours) could

restore Aβ-mediated inhibitory effects on pSer473 Akt and Nanog expressions in

miR-302-overexpressed cells (n = 3, p<0.05) (Fig. 4E), demonstrating that miR-302

activates Akt signaling to induce Nanog expression.

To determine whether Nanog plays a protective role in Aβ treatment, we further performed shRNA-mediated knockdown of Nanog in miR-302-overexpressed cells. As shown in Fig. 4F, downregulation of Nanog resulted in an increase of p-Ser307 IRS-1 expression as well as a decrease of both tyrosine phosphorylation and p-Ser 473-Akt/ p-Ser 9-GSK3β levels in miR-302-overexpressed cells after Aβ treatment. Taken together, our results strongly suggest that miR-302 may confer protection against Aβ-induced neurotoxicity by downregulating PTEN to activate Akt and the downstream Nanog signaling.

In vitro and in vivo gene expression levels of Naong and LARP7

We have observed that impaired Nanog expression is associated with Aβ-disrupted insulin sensitivity. To investigate this point, we performed RT-qPCR to show that Aβ treatment significantly decreased Nanog mRNA expression in control neurons in vitro (Fig. 5A). Next, we addressed the relevance of this finding to human AD patients in vivo by measuring the mRNA expression levels of Nanog in AD patients' PBMCs. A detailed overview of the testing subjects' characteristics is summarized in Table 1. A number of AD patients (n = 7) had moderated dementia by MMSE and CASI measurement scales, which can differentiate between AD patients and age-matched healthy controls (n = 6). As a result, both scales of MMSE and CASI

were decreased in these AD patients (Table. 1). Fig. 5B further showed that the level of Nanog mRNA was significantly decreased in AD patients compared to normal age-match controls. This observation confirmed our hypothesis that AD patients exhibit reduced Nanog expression, which may contribute to the pathogenesis of AD-associated neurodegeneration.

In addition, recent studies have indicated that miR-302 is encoded in the LARP7 gene on the chromosome 4 of human genome [33]. To determine whether the endogenous level of miR-302 was affected by Aβ-induced neurotoxicity during the progression of AD, we examined the expression of miR-302-encoding LARP7 gene by RT-qPCR with a special primer directed against the joining region of exons 8 and 9, as description in methods. Since Fig. 5C has shown that Aβ treatment markedly decreased LARP7 mRNA expression in control neurons in vitro, we further measured the mRNA expression of LARP7 in AD patients' PBMCs. As revealed in Fig. 5D, the expression of LARP7 mRNA was significantly decreased in AD patients compared to normal age-match controls. These results proved that the endogenous expression of miR-302 may play a role in the progression of AD, albeit the underlying mechanism remains unclear. Taken together, our findings clearly demonstrated that an impaired Nanog expression takes place in age-associated AD, which may serve as a novel diagnostic marker for AD.

DISCUSSION

Impairment of insulin signaling not only presents a serious threat to neuron survival but also plays a critical role in ageing-related diseases such as AD. Our study, for the first time, demonstrated that miR-302 regulates cell survival and anti-ageing processes via activating the Akt signaling pathway, which may confer protection against Aβ-induced neurotoxicity in human neuronal cells. We herein concluded that: (i) miR-302 silences PTEN to activate Akt signaling, which the stimulates Nrf2/HO-1 elevation and hence attenuates Aβ-induced apoptosis, and (ii) miR-302-mediated Akt activation also stimulates Nanog expression to suppress p-Ser307 IRS-1 expression and thus enhance IRS-1 tyrosine phosphorylation and p-Ser 473-Akt/ p-Ser 9-GSK3β formation. Conceivably, both of these newly identified miR-302 effects are useful for developing AD-related therapies.

We also found that miR-302-stimulated Akt signaling is able to attenuate many Aβ-associated AD symptoms, including neuronal insulin resistance, tau hyperphosphorylation, oxidative stress, and neuronal death. Our recent studies have reported that Aβ treatment causes tau hyperphosphorylation [8] through activating GSK3β [9] in SK-N-MC cells [8]. Moreover, the Aβ-induced upregulation of pSer307 IRS-1 has been shown to inhibit the insulin-downstream Akt/GSK3β signaling in

cognition-related brain areas, leading to brain insulin resistance [4]. As a result,

GSK3β activation regulates tau binding to microtubules and causes tau aggregation,

subsequently resulting in tau hyperphosphorylation [9] as well as neuronal death [8].

In contrast, upregulation of Akt has been reported reduce Aβ-induced neurotoxicity,

such as insulin resistance, tau hyperphosphorylation and neuronal death [8]. In view

of these previously established evidences, our results further indicated that miR-302

expression can stimulate Akt activation and hence may improve all these

Aβ-associated AD symptoms.

Aβ has been reported to trigger mitochondrial dysfunction and induce the

generation of ROS [31], both of which represent typical characteristics of ageing [5-7].

As oxidative stress is one of major causes for the onset of many degenerative

disorders and ageing [8], Aβ-induced ROS accumulation may eventually lead to

neuronal death. Interestingly, recent studies observed that miR-302 is able to inhibit

oxidant-induced cell death in adipose tissue-derived mesenchymal stem cell [19].

Similarly, we found that miR-302 can attenuate Aβ-induced oxidative stress through

activation of Nrf2 and HO-1. Nrf2 is a redox-sensitive transcription factor responsible

for regulating the induction of several important anti-oxidant enzymes, including

NAD(P)H quinone oxidoreductase 1, glutamate-cysteine ligase, and particularly HO-1

[23]. Since PI3K/Akt signaling has been reported to mediate Nrf2/HO-1 activities to

reduce cytotoxicity in oxidative stress-damaged neurons [24], it is conceivable that

Akt plays a pivotal role in regulating neuronal survival. To this, our results have

clearly demonstrated this point by showing that miR-302 activates Akt signaling to

upregulate Nrf2/HO-1 activities, so as to inhibit Aβ-induced oxidative stress and

maintain the intracellular redox balance toward cell survival.

Most importantly, our study revealed that miR-302 expression prevents

Aβ-induced neurotoxicity through activation of Akt-mediated Nanog expression. We

also found that miR-302 activates Akt/GSK3β signaling by knocking down a

PI3K/Akt inhibitor, PTEN. It has been known that miR-302 targets PTEN's mRNA 3′

UTR translational suppression [25, 34, 35]. This finding is also supported by a recent

study showing that miR-302 promotes human ESC pluripotency by knocking down

PTEN to activate Akt signaling [25]. PTEN is a well known negative regulator of

PI3K signaling and positively regulates insulin signaling [36]. In addition, PTEN is a

key negative regulator of PI3K/AKT signaling that is arguably the most important

pro-survival pathway and ageing-associated disease in neurons [37]. However, loss of

PTEN antagonizes the progression of ageing through promoting regeneration and

preventing oxidative stress-induced cell death [36, 38]. Recent studies have also

demonstrated that knockdown of PTEN results in a significant increase of the

pluripotency-associated gene Nanog expression [26]. Particularly, the PTEN-targeted

PI3K/Akt signaling has been shown to play an important role in maintaining iPSC status [39]. Nanog is a key determinant that maintains self-renewal of undifferentiated stem cells and hence its expression may be regulated by Akt signaling in differentiated cells [40]. To this, Chen *et. al.* have reported that retinol enhances the expression of Nanog, which directly activates IRS-1 by engaging Akt and preventing differentiation of ESCs [41]. Therefore, deciphering the underlying mechanisms of Nanog-mediated Akt signaling may have a wide impact on many biomedical research fields.

Taken together, in addition to numerous evidence showing that Akt/GSK3β are critical regulators of AD progression, our findings further indicated that miR-302 is able to target and downregulate PTEN to re-activate Akt/GSK3β signaling and Nanog expression, so as to prevent Akt/GSK3β-associated AD pathogenesis and progression. Furthermore, concurrent downregulation of Nanog and miR-302-encoding LARP7 gene expressions may serve as a diagnostic maker as well as a therapeutic target for AD. In light of our findings, it is conceivable that Akt/GSK3β signaling is associated with regulation of ageing, and hence may serve as a novel target to treat AD. Since Akt signaling is an important regulator for pluripotency and ageing-associated disorders [39], miR-302 expression may confer anti-apoptotic and anti-oxidative effects through activation of Akt signaling [15, 17, 25, 39]. In summary, we conclude

that an elevated expression of miR-302 prevents Aβ-induced neurotoxicity through activation of Akt/GSK3β signaling by downregulating PTEN and upregulating Nanog, as summarized in Fig. 6.

DISCLOSURE STATEMENT

No actual or potential conflict of interest.

FOOTNOTE

Author Contributions

Study conception and design: Hsin-Hua Li, Shi-Lung Lin, Te-Jen Lai, Chih-Li Lin

Acquisition of data: Hsin-Hua Li, Pai-Yi Chiu, Te-Jen Lai, Chih-Li Lin

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Figure 1. miR-302 attenuates Aβ-induced apoptosis in human SK-N-MC neuronal

cells.

(A) Transfection of SK-N-MC cells with either the AcGFP-LVX-miR-302 vector (+,

to form miR-302-overexpresed cells) or an empty vector (-, to form control cells),

using a lipofectamine 2000 reagent. Positively transfected cells were detected by

co-expression of a green fluorescent protein (AcGFP) under an inverted fluorescent

microscope. (B) RT-qPCR analyses of miR-302 expression using total RNA samples

extracted from miR-302-overexpressed (+) or control (-) cells, respectively. The

detected miR-302 expression levels in transfected cells were normalized with the

levels of control cells (n = 3, p<0.01). (C) Cell viability was determined by MTT

assays. Cells were seeded in 24-welled plates overnight and then treated with 2.5 μM

Aβ for 24 hrs. The results of cell viability were normalized using the level of control

cells, showing that ectopic miR-302 expression significantly reduced Aβ-induced cell

death. (D) Morphological changes of nuclear chromatins during apoptosis were

observed under fluorescent microscopy with DAPI staining. Cells were cultivated on

coated slides and treated with 2.5 μM Aβ for 24 hours. The nuclei fragmentation was

labeled (whit arrow) and was quantified by counting four random fields per condition

(bottom graph) (E) Aβ-induced cell apoptosis was determined by Western blotting of

cleaved caspase 3 and PARP proteins, respectively, after Aβ treatment (2.5 μM Aβ for

24 hours). The blotting results were normalized with the density level of control cells, showing marked decreases of Aβ-induced caspase 3 and PARP cleavage in miR-302-overexpressed cells (n = 3, p<0.01). (Aβ, β-amyloid; +, with treatment; -, without treatment. All values were presented as mean ± S.E.M. Significant differences were determined by multiple comparisons using Dunnett's *post-hoc trest for * p < 0.05 and ** p < 0.01.*)

Figure 2. miR-302 expression activates Akt and diminishes Aβ-induced cytotoxicity. (A) Western blot analyses of pSer307-IRS-1, pTyr-IRS-1, and pSer473-Akt expressions 24 hours after Aβ treatment (2.5 μM), showing marked elevation of pSer307-IRS-1 (n = 3, p<0.01) as well as reduction of both pTyr-IRS-1 and pSer473-Akt levels (n = 3, p<0.05) in control groups compared to those of miR-302-overexpressed cells. (B) Western blot analysis of pSer473-Akt levels after treatments of 2.5 μM Aβ or 20 μM LY294002, or both for 24 hours, (C) Cell viability in response to the treatments of (B), as determined by MTT assays. (D) Western blot measurement of pSer9-GSK3β, and pThr231-tau levels in response to the treatments of (B), showing that miR-302 overexpression stimulated Akt signaling to counteract Aß-mediated cytotoxicity, resulting in a marked increase of GSK3β Ser9 phosphorylation and decrease of tau-Thr231 phosphorylation (n = 3, p<0.05). Yet,

further co-treatment of Aβ (2.5 μM) and LY294002 (20 μM) abolished all these protective effects of Akt signaling in miR-302-overexpressed cells (n = 3, p<0.05). (Aβ, ß-amyloid. +, with treatment; -, without treatment. All values were presented as mean ± S.E.M. Significant differences were determined by multiple comparisons using Dunnett's *post-hoc trest for * p < 0.05 and ** p < 0.01.*)

Figure 3. miR302-induced Akt activation attenuates Aβ-induced oxidative stress.

(A) Intracellular superoxide radical anions stained with DHE were detected by fluorescence microscopy. Cells were treated with 2.5 μM Aβ or 1 μM insulin, or both, for 2 hours and then analyzed with DHE staining. The intensity of red fluorescent dye was normalized with the level of control cells before comparison. (B) After 24-hour Aβ treatment (2.5 μM), Western blot analyses showed that the expression of Nrf2 and HO-1 were decreased in control cells compared to those of miR-302-overexpressed cells (n = 3, p<0.05). (C) Cells were treated with 2.5 μM Aβ in the presence of 1 μM insulin or 20 μM LY294002, or both, and then analyzed with Western blotting for Nrf2. As shown, co-treatment of Aβ and LY294002 inhibited Nrf2 expression (n = 3, p<0.05), whereas further treatment with insulin (1 μM) prevented this inhibitory effect on Nrf2 expression (n = 3, p<0.05). (D) Cells of (C) were further stained with JC-1 dye and observed under an inverted fluorescent microscope, showing that Aβ

treatment reduced the intensity of JC-1 green fluorescence in miR-302-overexpressed cells (n = 3, p<0.05), while further treatment of LY294002 (20 μM) prevented this effect. (E) Western blotting analyses showing that a significant increase of tBid and decrease of Bcl-2 were observed in control cells compared to miR-302-overexpressed cells after 24-hour Aβ treatment (2.5 μM), (Aβ, ß-amyloid. +, with treatment; -, without treatment. For fluorescent density quantification, the levels of tested cells were normalized with that of control cells before comparison. Values were presented as mean ± S.E.M. Significant differences were determined by multiple comparisons using Dunnett's *post-hoc trest for * p < 0.05 and ** p < 0.01.*)

Figure 4. miR-302 targets PTEN and upregulated Nanog through Akt signaling.

(A) Alignment of predicted miR-302 binding sites within human PTEN 3'UTR was shown. (B,C) Cells lysates were obtained from untreated control cells and miR-302-overexpressed cells, respectively, and further analyzed with Western blotting for PTEN and Nanog, showing the downregulation of PTEN and upregulation of Nanog in miR-302-overexpressed cells (n = 3, p<0.05). (D) Western blot analyses of PTEN, pSer473 Akt, and Nanog expressions after 24-hour Aß treatment (2.5 μM), showing an increase of PTEN (p<0.05) and decreases of pSer473 Akt (p<0.05) and Nanog in control cells (n = 3, p<0.01) compared to those of miR-302-overexpression

cells (n = 3, p<0.05). (E) Western blot analyses of pSer473 Akt and Nanog expressions 24 hours after treatment of Aβ (2.5 μM) or LY294002 (20 μM), or both, showing that both pSer473 Akt and Nanog were significantly decreased in miR-302-overexpressed cells treated with both Aß and LY294002 (n = 3, p<0.05). (F) The miR-302-overexpressed cells were transiently transfected with shRNA-Nanog, and then treated with Aβ (2.5 μM) for 24 hours. shRNA-directed knockdown of Nanog markedly elevated pSer307-IRS-1 and reduced the levels of pTyr-IRS-1, pSer473-Akt and pSer9-GSK3β expressions in miR-302-overexpressed cells compared to those of control cells treated with Aβ alone. (Aβ, ß-amyloid. shRNA-Nanog, shRNA gene silencer directed against human Nanog. +, with treatment; -, without treatment. The results of density quantification were normalized with the level of control cells. Values were presented as mean ± S.E.M. Significant differences were determined by multiple comparisons using Dunnett's *post-hoc trest for * p < 0.05 and ** p < 0.01.*).

Figure 5. Comparison of the expression levels of *Naong* and *LARP7* mRNAs *in vitro* and *in vivo*.

(A) After 24-hour Aβ treatment (2.5 μM), the expression of *Nanog* mRNA was markedly decreased in control cells in vitro (n = 3, p<0.05). (B) Both AD patients' (n

= 7) and normal age-matched individual's (n = 6) blood samples were collected, separately, and total RNAs were then extracted and used for RT-qPCR analyses. The results showed that AD patients' PBMCs express significantly lower *Nanog* mRNAs than that of normal individuals (p<0.05). (C) Following 24-hour Aβ treatment (2.5 μM), the expression of LARP7 mRNA was markedly reduced in control cells compared to that of miR-302-overexpressed cells in vitro (n = 3, p<0.05). (D) AD patients' PBMCs expressed significantly lower LARP7 mRNA levels than that of normal individuals (p<0.05). (Aβ, ß-amyloid. AD, Alzheimer diseases. Levels of mRNA expression were normalized with the levels of control cells or normal healthy individuals. Values were presented as mean ± S.E.M. Significant differences were determined by using multiple comparisons of Dunnett's *post-hoc trest for * p < 0.05 and ** p < 0.01.*)

Figure 6. A proposed scheme for the protective effects of miR-302 against Aβ-induced neurotoxicity.

Upregulation of miR-302 can silence PTEN to activate Akt signaling, which subsequent (i) stimulates Nrf2/HO-1 elevation and hence attenuates Aβ-induced oxidative stress and apoptosis, and (ii) stimulates Nanog expression to suppress p-Ser307 IRS-1 expression, resulting in a significant increase of insulin/IRS-1/Akt

signaling, so as to inhibit GSK3β-mediated tau hyperphosphorylation.

Table 1. Data of AD patients and age-matched healthy individuals included in the study. The table presents gender, age, MMSE and CASI scores for AD patients and healthy individual controls, respectively.

Supplemental figure 1. Schematic overview of the miR-302-encoding LARP7 gene locus and the designed primers directed against both ends of the endogenous miR-302 familial cluster, of which the expression rates can be detected in proportional to the splicing levels of LARP7 mRNAs.

Supplemental table 1. The primer sequences used for RT-qPCR are listed.

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A.

miR-302   -   +

Bright field

Green fluorescence

30 µM

B.

miR-302 expression

Relative mRNA levels
(Normalized with control cells)

**

140000
120000
100000
80000
60000
40000

4
2
0

miR-302   -   +

Figure 1

C.

Cell viability

| miR-302 | - | - | + | + |
| Aβ (2.5 μM) | - | + | - | + |

D.

Figure 1

Figure 1

A.

Figure 2

B.

miR-302	-	-	+	+	+	-	+
Aβ (2.5 μM)	-	+	-	+	+	-	-
LY294002 (20 μM)	-	-	-	-	+	+	+

p-Ser^{473}-Akt

Akt

β-actin

p-Ser^{473}-Akt

Relative density (% of control cells)

miR-302	-	-	+	+	+	-	+
Aβ (2.5 μM)	-	+	-	+	+	-	-
LY294002 (20 μM)	-	-	-	-	+	+	+

Figure 2

C.

Cell viability

miR-302	-	-	+	+	+	-	+
Aβ (2.5 μM)	-	+	-	+	+	-	-
LY294002 (20 μM)	-	-	-	-	+	+	+

D.

miR-302	-	-	+	+	+
Aβ (2.5 μM)	-	+	-	+	+
LY294002 (20 μM)	-	-	-	-	+

p-Ser^9-GSK3β

GSK3β

p-Thr^{231}-Tau

Tau

β-actin

Figure 2

Figure 2

A.

miR-302 − +

control

Aβ (2.5 μM)

Aβ (2.5 μM)
Insulin (1 μM)

Insulin (1 μM)

40 μM

miR-302	−	−	−	−	+	+	+	+
Aβ (2.5 μM)	−	+	+	−	−	+	+	−
Insulin (1 μM)	−	−	+	+	−	−	+	+

% of control cells

** * *

Figure 3

B.

| miR-302 | - | - | + | + |
| Aβ (2.5 μM) | - | + | - | + |

Nrf2

HO-1

β-actin

Nrf2

Relative density
(% of control cells)

1.5
1.0
0.5
0.0

*   *

| miR-302 | - | - | + | + |
| Aβ (2.5 μM) | - | + | - | + |

HO-1

1.5
1.0
0.5
0.0

| miR-302 | - | - | + | + |
| Aβ (2.5 μM) | - | + | - | + |

C.

miR-302	-	-	-	-	+	+	+	+
Aβ (2.5 μM)	-	+	-	+	-	+	+	-
insulin (1 μM)	-	-	+	+	-	-	-	-
LY294002 (20 μM)	-	-	-	-	-	-	+	+

Nrf2

β-actin

Nrf2

Relative density
(% of control cells)

2.0
1.5
1.0
0.5
0.0

*   *   *

miR-302	-	-	-	-	+	+	+	+
Aβ (2.5 μM)	-	+	-	+	-	+	+	-
insulin (1 μM)	-	-	+	+	-	-	-	-
LY294002 (20 μM)	-	-	-	-	-	-	+	+

Figure 3

D.

miR-302   -   +

control

Aβ (2.5 μM)

Aβ (2.5 μM)
LY294002 (20 μM)

LY294002 (20 μM)

Figure 3

E.

Figure 3

A.

Human PTEN 3'UTR (3303 bp)

ORF

■ miR-302

Position 277-284 of PTEN 3'UTR
Seed match: 7mer-m8

B.
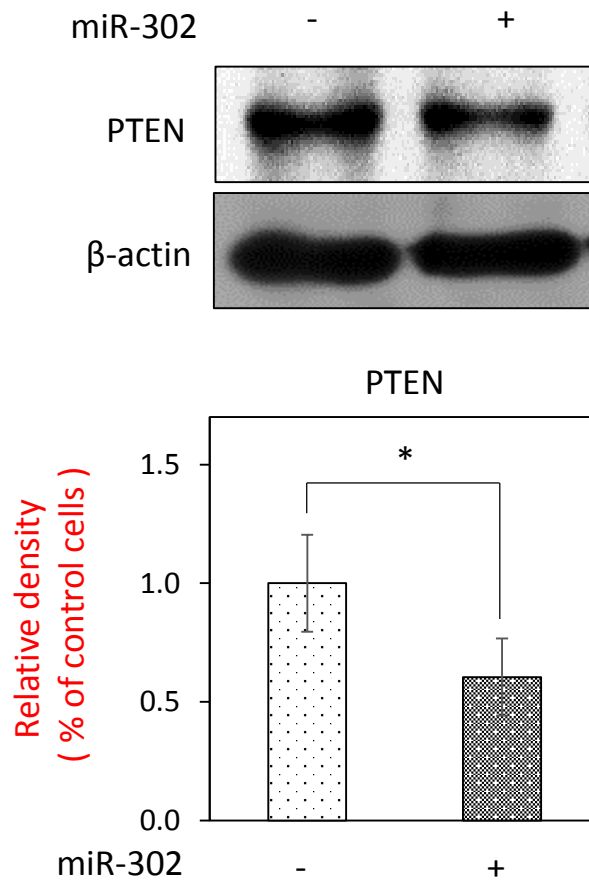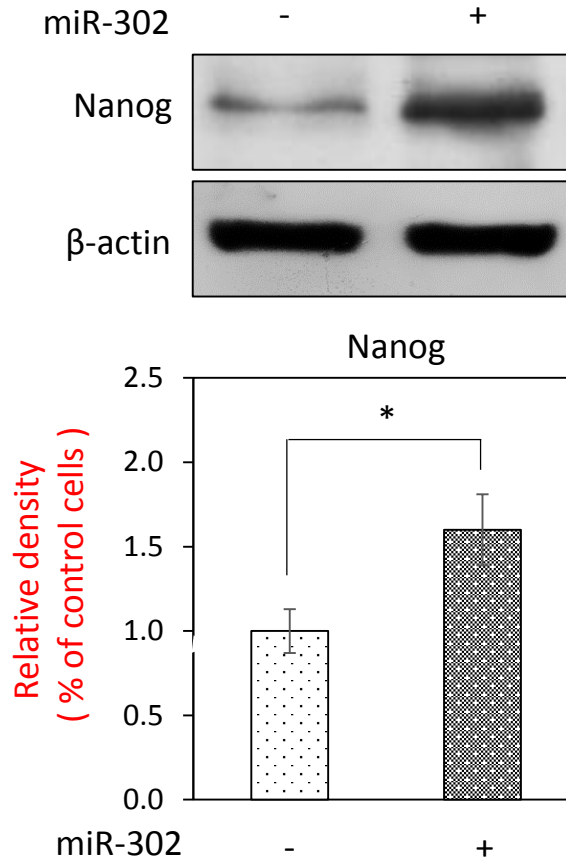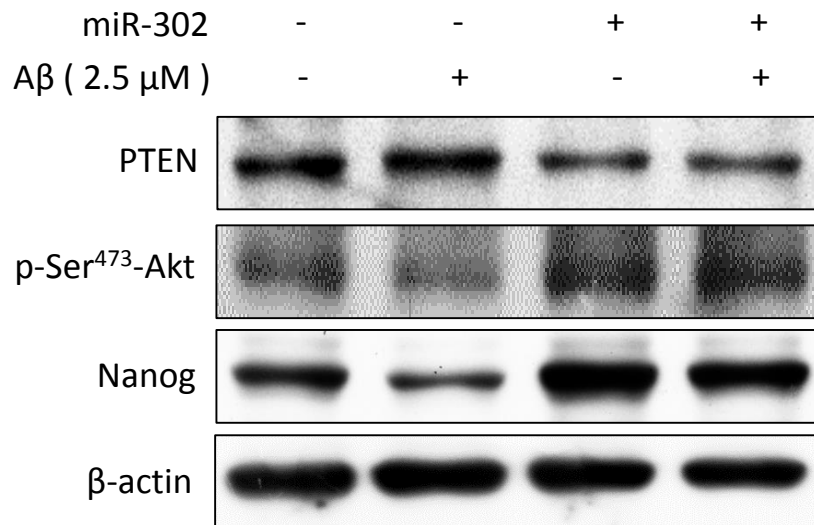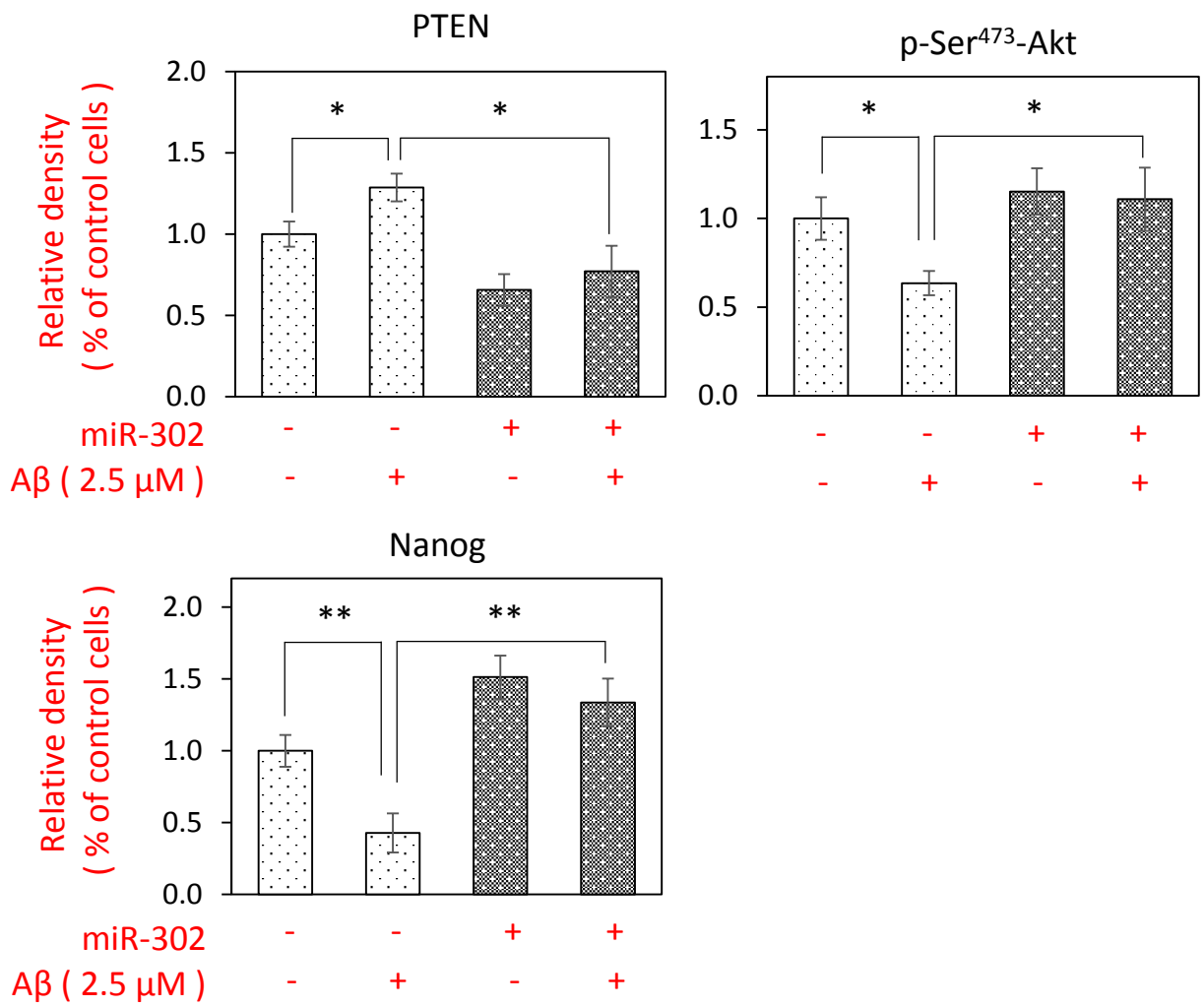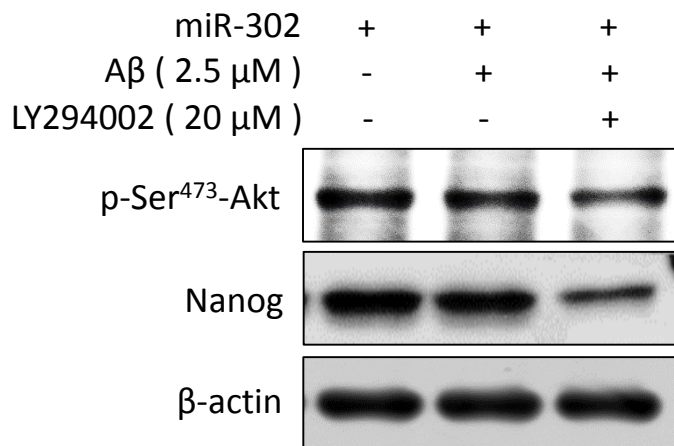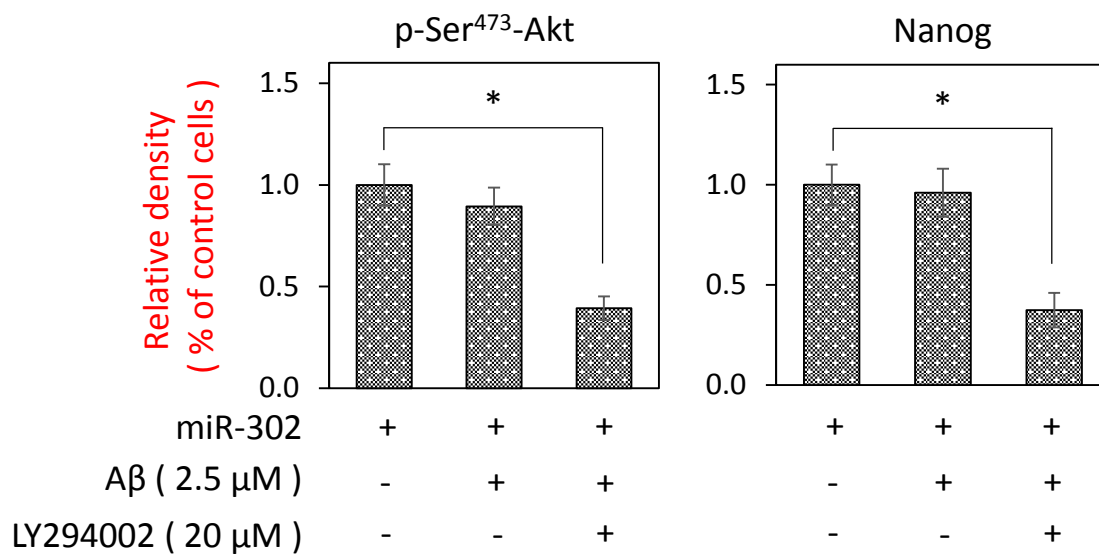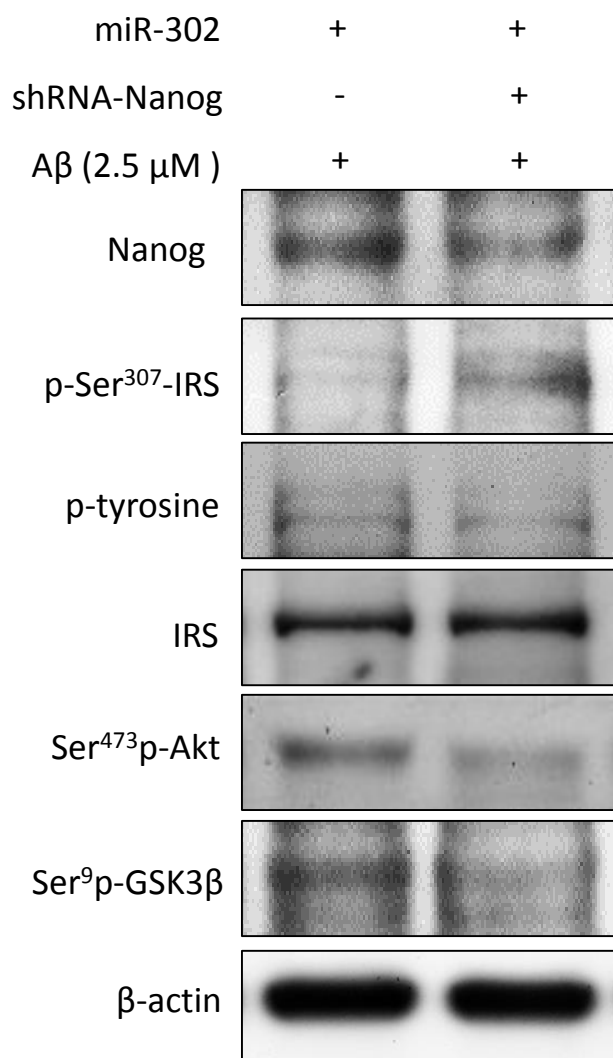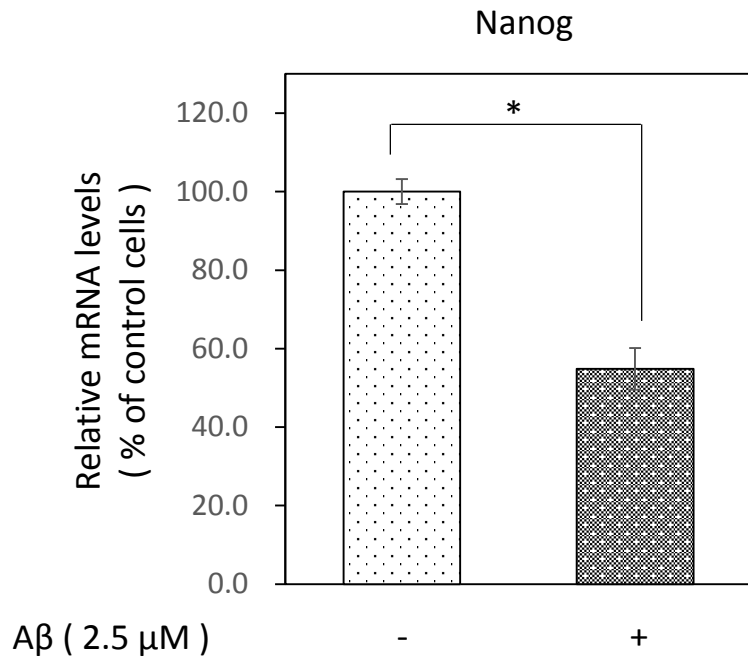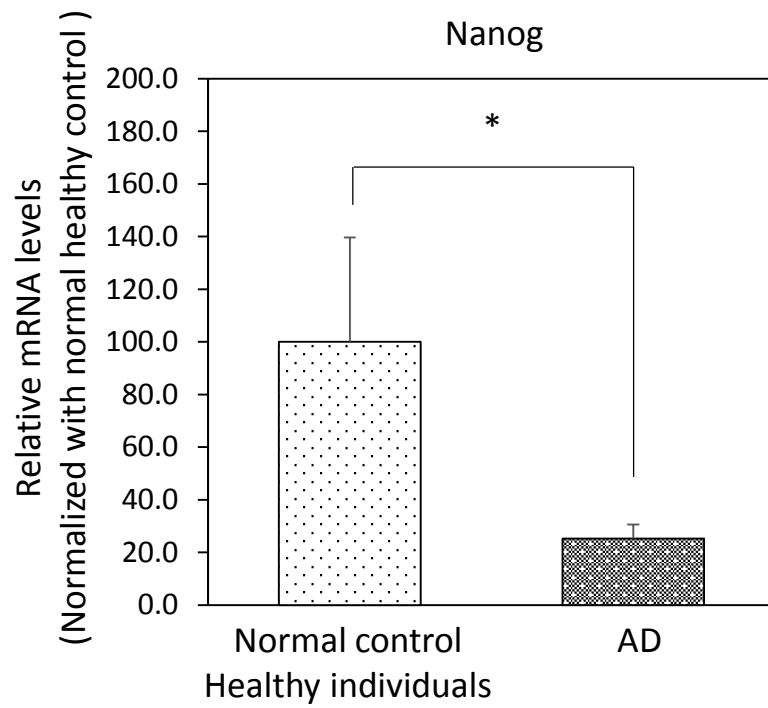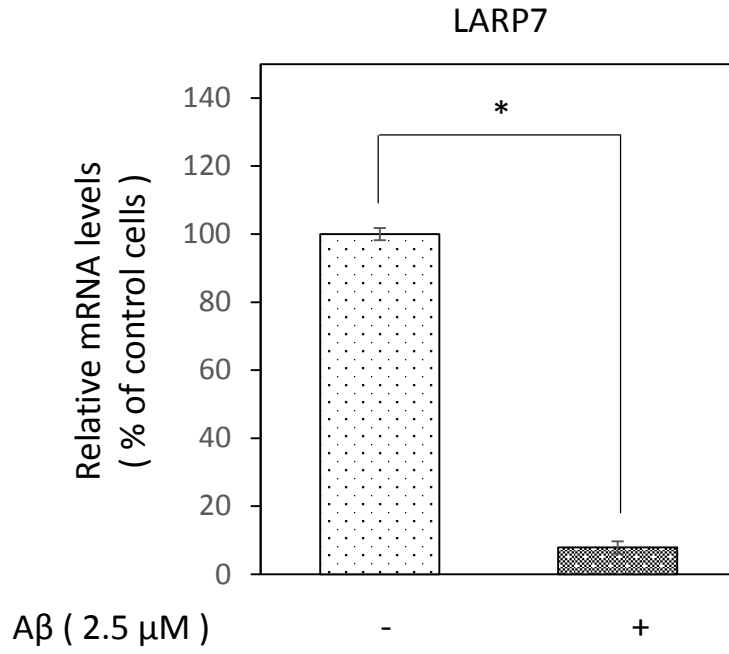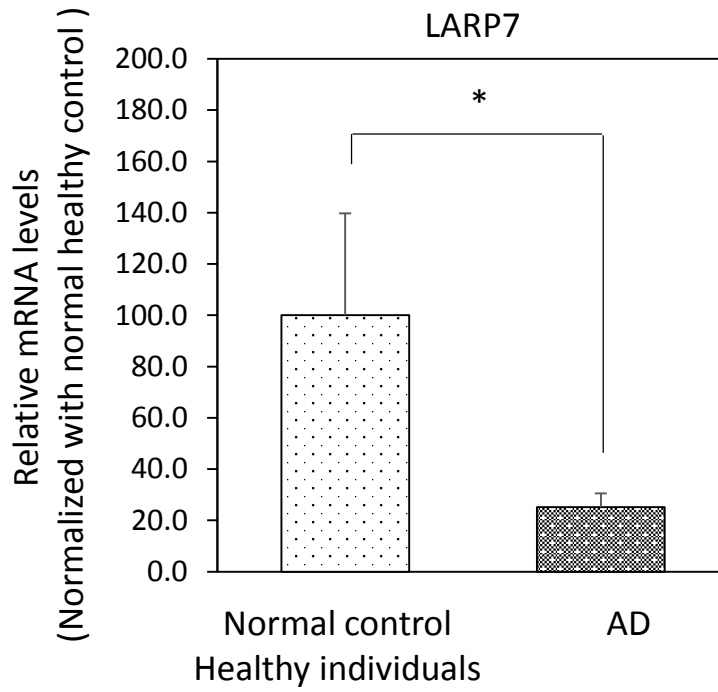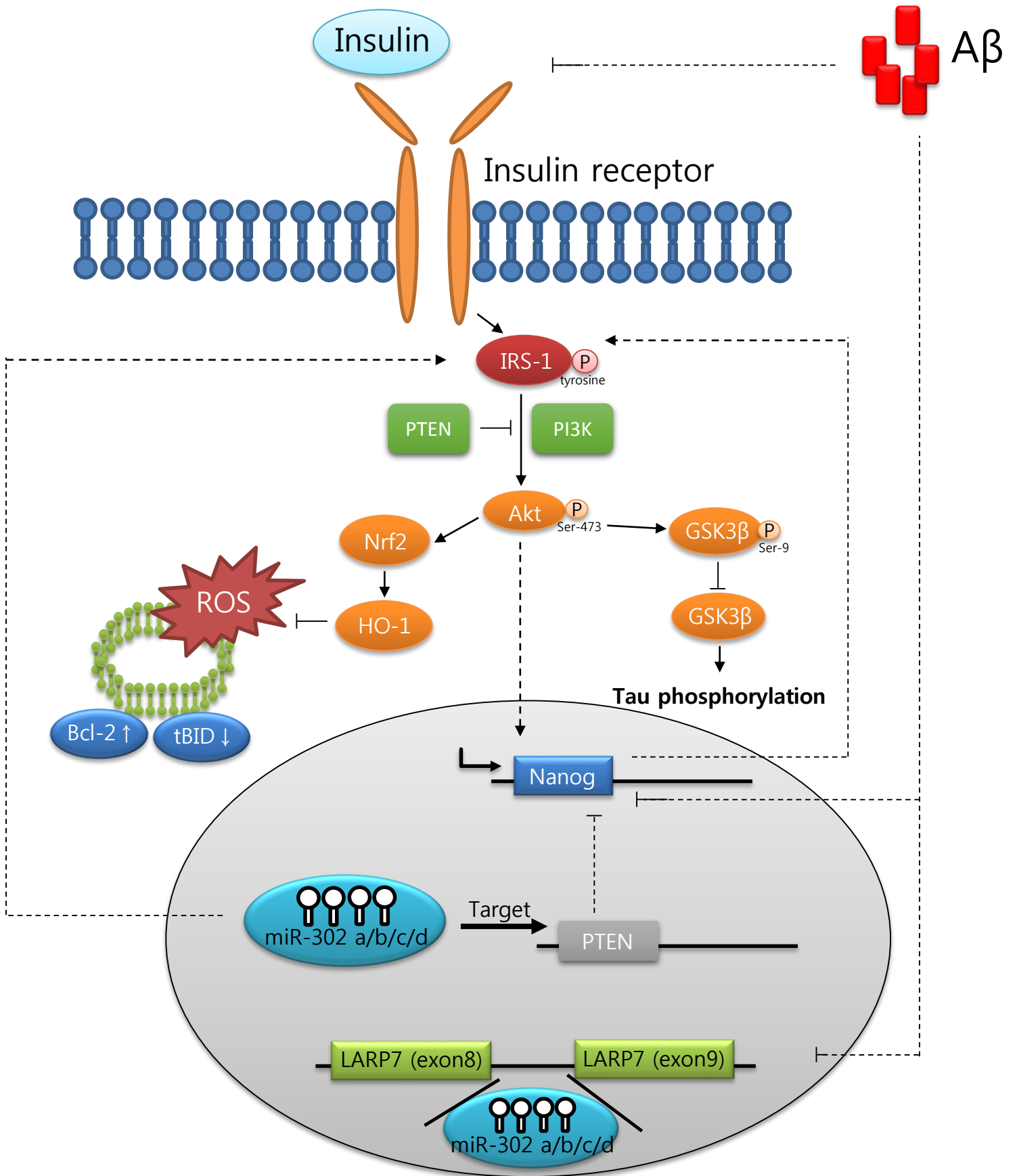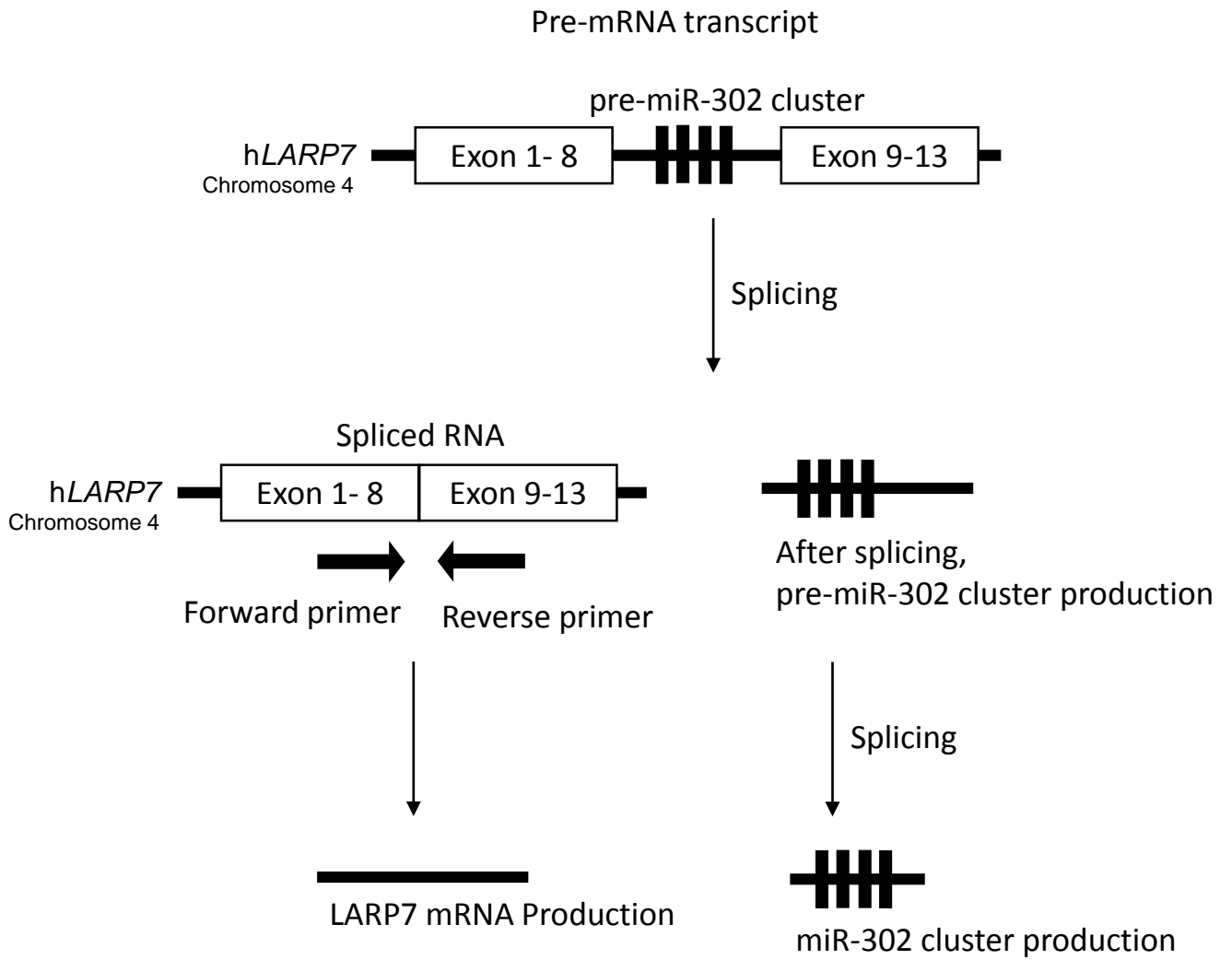
miR-302   −   +

PTEN

β-actin

PTEN

*

1.5

1.0

0.5

0.0

Relative density
(% of control cells)

miR-302   −   +

Figure 4

C.

miR-302   -   +

Nanog

β-actin

Nanog

D.

miR-302   -   -   +   +
Aβ (2.5 μM)   -   +   -   +

PTEN

p-Ser^{473}-Akt

Nanog

β-actin

Figure 4

PTEN

miR-302	-	-	+	+
Aβ (2.5 μM)	-	+	-	+

p-Ser473-Akt

miR-302	-	-	+	+
Aβ (2.5 μM)	-	+	-	+

Nanog

miR-302	-	-	+	+
Aβ (2.5 μM)	-	+	-	+

E.

miR-302	+	+	+
Aβ (2.5 μM)	-	+	+
LY294002 (20 μM)	-	-	+

p-Ser473-Akt

Nanog

β-actin

Figure 4

Figure 4

A.

Nanog

Relative mRNA levels (% of control cells)

120.0

100.0

80.0

60.0

40.0

20.0

0.0

*

Aβ (2.5 μM)   -   +

B.

Nanog

Relative mRNA levels (Normalized with normal healthy control)

200.0
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0

*

Normal control   AD
Healthy individuals

Figure 5

C.

LARP7

Aβ (2.5 μM)   -   +

D.

LARP7

Figure 5

Figure 6

Pre-mRNA transcript

pre-miR-302 cluster

h*LARP7*
Chromosome 4

Exon 1- 8

Exon 9-13

Splicing

Spliced RNA

h*LARP7*
Chromosome 4

Exon 1- 8

Exon 9-13

Forward primer

Reverse primer

After splicing,
pre-miR-302 cluster production

Splicing

LARP7 mRNA Production

miR-302 cluster production

Supplementary figure 1

miR-302	−	+
control		
Aβ (2.5 μM)		
Aβ (2.5 μM) Insulin (1 μM)		
Insulin (1 μM)		
Aβ (2.5 μM) LY294002 (20 μM)		
LY294002 (20 μM)		

40 μM

Supplementary figure 2

| miR-302 | − | + |

control

Aβ (2.5 µM)

Aβ (2.5 µM)
LY294002 (20 µM)

LY294002 (20 µM)

Aβ (2.5 µM)
insulin (1 µM)

insulin (1 µM)

Supplementary figure 3

Title: Epigallocatechin gallate attenuates amyloid β-induced inflammation and neurotoxicity in EOC 13.31 microglia

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Abstract: Microglia are the primary immune cells that contribute to neuroinflammation by releasing various proinflammatory cytokines and neurotoxins in the brain. Microglia-mediated neuroinflammation is one of the key characteristics of Alzheimer's disease (AD). Therefore, inhibitory reagents that prevent microglial activation may be used as potential therapeutic agents for treating AD. Recently, many studies have been performed to determine the bioactivities of green tea polyphenol epigallocatechin-3-gallate (EGCG), an efficient antioxidant that prevents neuroinflammation. However, limited information is available on the effects of EGCG on microglia-mediated neuroinflammation. In this study, we investigated the inhibitory effects of EGCG on amyloid β (Aβ)-induced microglial activation and neurotoxicity. Our results indicated that EGCG significantly suppressed the expression of tumor necrosis factor α (TNFα), interleukin-1β, interleukin-6, and inducible nitric oxide synthase (iNOS) in Aβ-stimulated EOC 13.31 microglia. EGCG also restored the levels of intracellular antioxidants nuclear erythroid-2 related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), thus inhibiting reactive oxygen species-induced nuclear factor-κB (NF-κB) activation after Aβ treatment. Furthermore, EGCG effectively protected neuro-2a neuronal cells from Aβ-mediated, microglia-induced cytotoxicity by inhibiting mitogen-activated protein kinase-dependent, Aβ-induced release of TNFα. Taken together, our findings suggested that EGCG suppressed Aβ-induced neuroinflammatory response of microglia and protected against indirect neurotoxicity. These results suggest that EGCG is a possible therapeutic agent for preventing Aβ-induced inflammatory neurodegeneration.

Epigallocatechin gallate attenuates amyloid β-induced inflammation and neurotoxicity in EOC 13.31 microglia

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Abstract

Microglia are the primary immune cells that contribute to neuroinflammation by releasing various proinflammatory cytokines and neurotoxins in the brain. Microglia-mediated neuroinflammation is one of the key characteristics of Alzheimer's disease (AD). Therefore, inhibitory reagents that prevent microglial activation may be used as potential therapeutic agents for treating AD. Recently, many studies have been performed to determine the bioactivities of green tea polyphenol epigallocatechin-3-gallate (EGCG), an efficient antioxidant that prevents neuroinflammation. However, limited information is available on the effects of EGCG on microglia-mediated neuroinflammation. In this study, we investigated the inhibitory effects of EGCG on amyloid β (Aβ)-induced microglial activation and neurotoxicity. Our results indicated that EGCG significantly suppressed the expression of tumor necrosis factor α (TNFα), interleukin-1β, interleukin-6, and inducible nitric oxide synthase (iNOS) in Aβ-stimulated EOC 13.31 microglia. EGCG also restored the levels of intracellular antioxidants nuclear erythroid-2 related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), thus inhibiting reactive oxygen species-induced nuclear factor-κB (NF-κB) activation after Aβ treatment. Furthermore, EGCG effectively protected neuro-2a neuronal cells from Aβ-mediated, microglia-induced cytotoxicity by inhibiting mitogen-activated protein kinase-dependent, Aβ-induced release of TNFα. Taken together, our findings suggested that EGCG suppressed Aβ-induced neuroinflammatory response of microglia and protected against indirect neurotoxicity. These results suggest that EGCG is a possible therapeutic agent for preventing Aβ-induced inflammatory neurodegeneration.

Key words: microglia, amyloid β, epigallocatechin gallate, tumor necrosis factor α,

neuroinflammation

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and is characterized by progressive cognitive impairment and neuronal loss. Amyloid hypothesis suggests that amyloid β (Aβ) accumulation in the brain is a principal pathological feature of AD. Aβ is an important inducer of microglial activation and elicits chronic inflammation, which contributes to neurodegeneration and ultimately neuronal cell death (Khandelwal et al., 2011). Microglia are resident immune cells of the central nervous system and play a pivotal role in neuroinflammation (Broussard et al., 2012). Microglia are widely present in the brain, mainly the hippocampus and substantia nigra, and the spinal cord (Venneti et al., 2009), suggesting that microglial deterioration contributes to some neurodegenerative diseases. Prolonged exposure to stress signals overactivates microglia, which in turn induces neuroinflammation (Gonzalez et al., 2014). Under physiological conditions, microglia are usually present in a resting state. However, Aβ deposition in the brain activates microglia and increases their proliferation and secretion of various proinflammatory cytokines, including interleukin-1β (IL-1β) and IL-6 (Park et al., 2013b). In addition, increasing evidence indicates that tumor necrosis factor α (TNFα) is involved in microglia-mediated neurotoxicity, neuronal dysfunction, and neuronal cell death (Olmos and Llado, 2014). Moreover, Aβ stimulates *de novo* synthesis and TNFα release, which promotes neurotoxicity through microglial inflammatory pathways (Li et al., 2013). This suggests that TNFα plays a central role in microglia-mediated neuroinflammation and AD pathogenesis. In addition to releasing inflammatory cytokines, overactivated microglia synthesize and release some cytotoxic factors such as nitric oxide and reactive oxygen species, which cause significant neuronal cell damage (Mosher and Wyss-Coray, 2014). This suggests that microglia play a key role in the pathogenesis of neurodegenerative disorders such as AD. This indicates that

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specific agents downregulating microglia-mediated inflammation can exert potential therapeutic effects in patients with AD.

Several studies have been performed on epigallocatechin-3-gallate (EGCG), a major polyphenol isolated from green tea that exerts neuroprotective effects against Aβ-induced neurotoxicity (Queen and Tollefsbol, 2010). EGCG is the most abundant catechin in green tea and exhibits potent antioxidant and anti-inflammatory properties both *in vitro* and *in vivo* (Riegsecker et al., 2013). EGCG exerts neuroprotective effects against various disease conditions (Mandel et al., 2008); these include anti-amyloidogenic effects (Hyung et al., 2013), inhibition of endoplasmic reticulum stress (Yao et al., 2014), anti-neuroinflammatory effects (Herges et al., 2011), inhibition of stress-induced mitogen-activated protein kinase (MAPK) pathways (Kamalden et al., 2012), and suppression of oxidative and nitrosative cell death (Kim et al., 2009). These observations indicate that the neuroprotective effects of EGCG are attributed to its antioxidant activity and its ability to alter signaling pathways involved in neuronal damage. EGCG remodels Aβ fibrils into an unstructured conformation (Ehrnhoefer et al., 2008; Palhano et al., 2013); this significantly decreases the cellular toxicity of exogenous Aβ (Bieschke et al., 2010). Moreover, our previous study showed that EGCG suppressed Aβ-induced neurotoxicity by interrupting c-Abl signaling (Lin et al., 2009), which is involved in gliosis, neuroinflammation, and neuronal loss (Schlatterer et al., 2011). However, limited information is available on the effects of EGCG on Aβ-induced inflammatory responses in microglia. Therefore, in the present study, we investigated the preventive effects of EGCG on the neuroinflammatory responses of Aβ-stimulated microglia. In addition, we explored the molecular mechanisms underlying EGCG-inhibited microglial activation by examining the role of TNFα-induced MAPK signaling pathways. Our findings suggested that EGCG exerted therapeutic effects by inhibiting Aβ-induced microglial

inflammation and neurotoxicity.

2. Materials and methods

2.1. Materials

(-)-EGCG, *N*-acetyl-L-cysteine (NAc),

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT),

lipopolysaccharide (LPS) from *Escherichia coli* (serotype O55:B5), and

2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma (München,

Germany). MAPK-specific inhibitors SP600125, SB203580, and PD98059 were

obtained from Calbiochem (San Diego, CA, USA). Anti-β-actin antibody was

obtained from Novus Biologicals. (Littleton, CO, USA), and antibodies against p38,

phosphorylated p38 (Thr^{180}/Tyr^{182}), JNK, and phosphorylated JNK (Thr183/Tyr185)

were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti- inducible

nitric oxide synthase (anti-iNOS) antibody was purchased from Alexis Biochemicals

(San Diego, CA, USA), and antibodies against IκB-α, phosphorylated IκB-α, NF-κB

p65, nuclear erythroid-2 related factor 2 (Nrf2), heme oxygenase-1 (HO-1), ERK1/2,

phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}), and nitrotyrosine were purchased from Santa

Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse TNFα antibody was

purchased from R&D Systems (Minneapolis, MN, USA). $Aβ_{1-42}$ was purchased from

AnaSpec Inc. (San Jose, CA, USA), and Aβ solution was prepared as described

previously (Li et al., 2012). Briefly, 1 mM Aβ peptide was dissolved in 100%

1,1,1,3,3,3-hexafluoro-2-propanol and was dried using a vacuum desiccator. Next, 5

mM Aβ was resuspended in dimethylsulfoxide (DMSO) and stored at −20°C. To

obtain oligomers, Aβ peptide was diluted to a final concentration of 100 μM by using

Dulbecco's modified Eagle's medium (DMEM; Gibco), incubated at 4°C after 24 h of

gentle shaking, and immediately added to cell cultures at a final concentration of 10

μM.

2.2 Cell culture and viability assay

EOC 13.31 mouse immortalized microglia (ATCC CRL-2468) were obtained from

American Type Culture Collection (Bethesda, MD, USA). Neuro-2a mouse

neuroblastoma cells (BCRC 60026) were purchased from Bioresources Collection and

Research Center (BCRC, Hsinchu, Taiwan). Both the cell lines were maintained in

DMEM supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL

streptomycin, and 2 mM L-glutamine and incubated at 37°C in a humidified

atmosphere of 5% CO_2. The cell viability was assessed by performing MTT-based

colorimetric assay. In brief, the cells were seeded overnight in 24-well plates at a

density of 5×10^4 cells/well and treated as indicated. After 24 h, tetrazolium salt MTT

was added to the medium. The supernatant was discarded, and the cells were

solubilized using DMSO. Only viable cells reduce MTT metabolically to a purple

formazan product, which was quantified spectrophotometrically at 550 nm by using a

microplate reader. Cell viability was expressed as the percentage of control cells

treated with vehicle alone.

2.3 Real-time PCR of mRNA expression

After the treatment, total mRNA was extracted using RNeasy Kit (Qiagen,

Germantown, MD, USA) and was quantified spectrophotometrically. The mRNA was

reverse transcribed to cDNA by using TProfessional Thermocycler (Biometra) under

the following conditions: primer binding at 25°C for 10 min, reverse transcription at

37°C for 120 min, and reverse transcriptase denaturation at 85°C. The mRNA was

quantified by performing real-time PCR (qPCR) by using ABI 7300 Sequence

Detection System (Applied Biosystems, Foster City, CA, USA). Amplifications of

target genes was performed using Power SYBR Green PCR Master Mix (Applied

Biosystems), according to the manufacturer's instructions. Each cDNA sample was

tested in triplicate. The following temperature parameters were used: initial

denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and

annealing at 60°C for 1 min and dissociation stage involving 95°C for 15 s, 60°C for

15 s, and 95°C for 15 s. The following primer pairs were used: forward

5′-AGGCACTCCCCCAAAAGATG-3′ and reverse

5′-TGAGGGTCTGGGCCATAGAA-3′ for TNFα, forward

5′-AGGGCTCTTCGGCAAATGTA-3′ and reverse

5′-GAAGGAATGCCCATTAACAACAA-3′ for IL-6, forward

5′-GCCCATCCTCTGTGACTCAT-3′ and reverse

5′-AGGCCACAGGTATTTTGTCG-3′ for IL-1β, forward

5′-GCGTTACTCCACCAACAATGGCAA-3′ and reverse

5′-ATAGAGGATGAGCTGAGCATTCCA-3′ for iNOS, and forward

5′-TGGTATCGTGGAAGGACTCATGAC-3′ and reverse

5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′ for GAPDH. Values of relative mRNA

expression were obtained by using SDS version 1.2.3 (Sequence Detection Systems

1.2.3-7300 Real Time PCR System; Applied Biosystems), and these values were

standardized by comparing with those for the relative expression of GAPDH. The

efficiency of qPCR results was found to be between 90% and 100% ($-3.6 \geq$ slope $\geq$

-3.3).

2.4 ELISA for determining TNFα, IL-6, and IL-1β levels

The cells were seeded overnight in 24-well plates at a density of 5×10^4 cells/well

and treated as indicated. TNFα, IL-6, and IL-1β levels in the culture media were

quantified using an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to

the manufacturer's instructions.

2.5 Measurement of reactive oxygen species levels

To evaluate the levels of intracellular reactive oxygen species, the cells were treated

with 10 μM dichlorodihydrofluorescein diacetate (DCFH-DA), a general oxidative

stress indicator, for 0.5 h at 37°C under 5% CO_2. The cells were then harvested and

washed twice immediately with PBS. Fluorescence intensity was determined using a

fluorescence microscope (DP72/CKX41; Olympus), and the cells were measured by

flow cytometry (FACSCalibur; BD Biosciences) at excitation and emission

wavelengths of 488 and 525 nm. Relative fluorescence intensity was obtained by

averaging the values obtained from three repeated experiments.

2.6 Western blotting

The cells were harvested and homogenized in a protein extraction lysis buffer [50 mM

Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% nonidet P-40, 0.5 mM

phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol] and nuclear extraction

reagent (Thermo scientific) to obtain cytosolic and nuclear extracts, respectively. Next,

the lysates were centrifuged at 12,000 $\times g$ for 30 min at 4°C. The supernatants were

used as cell extracts for immunoblotting analysis. SDS-solubilized samples were

loaded onto SDS-polyacrylamide gels. Total cell lysates containing equal amounts of

proteins were resolved by performing SDS-PAGE on 10% or 8% gels, transferred

onto polyvinylidene difluoride membranes (Millipore), and probed using primary

antibodies followed by horseradish peroxidase-conjugated secondary antibodies.

Immunocomplexes were visualized using enhanced chemiluminescence kits

(Millipore). Primary antibodies were used at a dilution of 1:1000 in 0.1% Tween 20,

and secondary antibodies were used at dilution of 1:5000.

2.7 Transient transfection of NF-κB luciferase reporter plasmid

EOC 13.31 cells were transfected with a luciferase reporter plasmid containing NF-κB

responsive element upstream of a firefly luciferase gene. After 18–24 h of transfection,

the cells were treated with the indicated compounds for 24 h. Cell lysates were

prepared using reporter lysis buffer (Promega Life Sciences), and luciferase activity

was measured using SpectraMax M5 Multi-Mode Microplate Reader (Molecular

Devices). The total amount of protein was determined by performing Bradford assay

(Bio-Rad) and was used to normalize the samples.

2.8 Collection of microglia conditioned medium and transferase-mediated

dUTP-biotin nick end-labeling assay

EOC 13.31 microglia were stimulated with 10 μM Aβ in the presence or absence of

10 μM EGCG or 100 ng/mL anti-mouse TNFα antibody for 24 h and were centrifuged

to obtain a cell-free supernatant. Neuro-2a neuronal cells were serum-starved for 4 h

and treated with 50% EOC 13.31 microglia-conditioned medium and 50% fresh

DMEM. After 24 h of incubation, the viability of neuro-2a cells was determined by

performing the MTT assay and transferase-mediated dUTP-biotin nick end-labeling

(TUNEL) assay. The TUNEL assay (Invitrogen) was performed according to the

manufacturer's instructions. For the TUNEL assay, neuro-2a cells were plated in

24-well plates, incubated in the microglia-conditioned medium for 48 h with either

protein samples or a control solution, fixed in 4% paraformaldehyde for 30 min at

37°C, and treated with DNase for 15 min.

2.9 Statistical analysis

All data were obtained from at least three independent experiments and were

expressed as mean ± standard error of the mean (S. E. M.). All statistical analyses were performed using analysis of variance followed by Dunnett's post-hoc test for multiple comparisons by using SPSS statistical software (SPSS, Inc., Chicago, IL, USA). The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 EGCG suppresses Aβ-induced increase in several neuroinflammatory molecules in EOC 13.31 microglia

EOC 13.31 is a highly proliferating microglial cell line that produces several cytokines contributing to the pathogenesis of AD. EOC 13.31 cells are not genetically modified and are highly similar to primary microglia (Stansley et al., 2012). Therefore, we used EOC 13.31 cells in most of our subsequent experiments. To investigate the cellular compatibility of EGCG with EOC 13.31 microglia, we performed the MTT cell viability assay by using different EGCG concentrations from 5 to 20 μM in the presence or absence of $A\beta_{1-42}$ for 24 h. EGCG did not exert significant toxic effects on cultured EOC 13.31 cells (Fig 1A). However, the cotreatment of EOC 13.31 cells with EGCG and 10 μM $A\beta_{1-42}$ slightly attenuated cytotoxicity; however, the effect was not statistically significant. Therefore, this concentration range of EGCG was used in our subsequent experiments. Studies indicate that Aβ stimulates microglia to produce and release proinflammatory cytokines. To determine the effects of EGCG on Aβ-induced neuroinflammation, we investigated whether EGCG attenuated the expression of proinflammatory molecules by EOC 13.31 cells. First, we examined whether bacterial endotoxin LPS or Aβ activated microglia. The production of proinflammatory cytokines such as TNFα, IL-6, and IL-1β as well as the neuroinflammation-related enzyme iNOS was markedly elicited in both LPS- or Aβ-activated microglia (Fig 1B). However, the treatment of microglia with 20 μM

EGCG significantly inhibited Aβ-induced mRNA expression of genes encoding proinflammatory cytokines. ELISA also showed that the levels of TNFα, IL-6, and IL-1β significantly decreased in Aβ-stimulated microglia compared with those in unactivated microglia (Fig. 1C). Collectively, these results suggested that EGCG suppressed the expression and secretion of neuroinflammatory molecules by Aβ-stimulated EOC 13.31 microglia.

3.2 EGCG inhibits reactive oxygen species-induced NF-κB activation and restores Nrf2/HO-1 antioxidant signaling

Recent evidence indicates that Aβ increases neuroinflammation and neurotoxicity through reactive oxygen species-dependent microglial activation (Parajuli et al., 2013). Because reactive oxygen species play an important role in oxidative damage-induced neurodegenerative diseases, we examined whether EGCG decreased Aβ-induced reactive oxygen species production in EOC 13.31 microglia. We used DCFH-DA fluorescent dye to measure reactive oxygen species production by performing fluorescence microscopy and flow cytometry. Aβ significantly stimulated reactive oxygen species generation in microglia (Fig. 2A). However, the cotreatment of EOC 13.31 microglia with EGCG and Aβ attenuated Aβ-induced reactive oxygen species production, as determined by performing fluorescence microscopy. EGCG significantly suppressed Aβ-induced reactive oxygen species production in a dose-dependent manner, and 20 μM EGCG exhibited the highest effect on the inhibition of reactive oxygen species production, with a 54.3% reduction at 24 h (Fig. 2B). However, treatment with up to 30 μM EGCG alone did not significantly affect basal reactive oxygen species levels (data not shown). Reactive oxygen species -induced microglial inflammation is mediated by the NF-κB pathway (Yuan et al., 2014). To determine whether NF-κB inhibition contributed to the anti-inflammatory

effect of EGCG, we examined the degradation of IκB and intracellular localization of NF-κB subunit p65. Aβ markedly increased the phosphorylation of IκB-α, decreased the expression of IκB-α and p65 in the cytosol, and increased the levels of p65 in the nucleus (Fig. 3C). However, EGCG significantly reversed the effects of Aβ on IκB-α and p65. To further verify the role of NF-κB in Aβ-activated microglia, we examined the effect of EGCG on Aβ-activated NF-κB signaling by performing a luciferase reporter assay. Our results showed that Aβ significantly induced the DNA-binding activity of NF-κB in the microglia (Fig. 2D), whereas EGCG dose dependently suppressed Aβ-induced activation of the NF-κB pathway. Antioxidant NAc inhibits Aβ-induced activation of NF-KB. These results suggested that EGCG inhibited reactive oxygen species-induced NF-κB activation in EOC 13.31 microglia after exposure to Aβ. Increased Nrf2 and HO-1 expression may exert detoxifying/antioxidant effects on microglia (Park et al., 2013a). We observed that the expression of both Nrf2 and HO-1 was suppressed in Aβ-treated cells but not in Aβ- and EGCG-cotreated cells (Fig. 2E). Taken together, our findings suggested that EGCG protected against Aβ-induced reactive oxygen species accumulation and NF-κB activation in EOC 13.31 microglia.

3.3 EGCG inhibits Aβ-induced activation of JNK, p38, and ERK1/2 MAPK signaling
MAPK signaling is important for the release of proinflammatory cytokines such as TNFα from microglia (Kaminska et al., 2009). Because 3 components of the MAPK family (JNK, p38, and ERK1/2) are implicated in the transcription and translation of proinflammatory factors, we investigated whether EGCG inhibited MAPK signaling in Aβ-stimulated microglia. EOC 13.31 cells were cotreated with different concentrations of EGCG and Aβ for 24 h. EGCG markedly inhibited Aβ-induced phosphorylation of JNK and p38 without affecting the total concentration of

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corresponding kinases (Fig. 3A and 3B). However, EGCG did not significantly affect Aβ-induced ERK phosphorylation (Fig. 3C). Moreover, we examined the inhibition of Aβ-induced upregulation of TNFα mRNA expression by using specific inhibitors of MAPK pathways, including SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), and PD98059 (ERK1/2 inhibitor). Similar to EGCG, the 3 inhibitors significantly reduced the amounts of TNFα mRNA expression in Aβ-treated microglia (Fig. 3D). This confirmed our hypothesis that the activation of MAPK pathways was involved in the upregulation of TNFα in Aβ-treated microglia, indicating that the suppression of Aβ-induced inflammatory response by EGCG was associated with the inhibition of MAPK signaling in EOC 13.31 microglia.

3.4 EGCG protects neuro-2a neuronal cells against Aβ-induced microglia-mediated cytotoxicity

The neuroinflammatory molecules released by activated microglia enhance Aβ-induced neurotoxicity and TNFα-induced neuronal apoptosis is involved in this process (Maccioni et al., 2009). Therefore, we examined whether EGCG played a direct protective role in Aβ-induced apoptosis of neuro-2a neuronal cells. Treatment with EGCG for 24 h did not significantly decrease Aβ-induced apoptosis of neuro-2a neuronal cells (Fig. 4A), suggesting that EGCG did not protect neuro-2a cells against Aβ-induced toxicity. Then, we investigated whether conditioned media collected from Aβ-stimulated microglia induced neuronal cell death. For this, we conducted the TUNEL assay to detect apoptosis and MTT assay to assess cell viability. Treatment of neuro-2a cells with conditioned media from Aβ-stimulated EOC 13.31 microglia significantly increased apoptotic cell death. However, treatment of neuro-2a cells with conditioned media from EOC 13.31 microglia cotreated with Aβ and EGCG decreased the numbers of TUNEL-positive cells. These results were corroborated by

the results of the MTT cell viability assay (Fig. 4B and 4C). To determine whether

TNFα was required for neuronal apoptosis, conditioned media from Aβ-stimulated

microglia were preincubated with neutralizing antibodies against mouse TNFα.

Preincubation with the antibodies significantly attenuated apoptosis induced by the

conditioned media (Fig. 4B and 4C). However, direct treatment of neuro-2a cells with

recombinant mouse anti-TNFα antibody had no effect on their survival (data not

shown). These data indicated that TNFα contributed to the neuronal cell death

observed in these cultures. Finally, we observed that neuro-2a cells treated with

conditioned media from Aβ-stimulated microglia showed increased nitrotyrosine

immunoreactivity compared with untreated controls and that EGCG prevented this

Aβ-induced iNOS overexpression and nitrotyrosine-modified protein formation, as

determined by performing western blotting of the lysates of the treated cells (Fig. 4D).

Overall, these results suggested that TNFα was a key player in microglia-mediated

neurotoxicity, whereas EGCG protected neuro-2a cells against indirect toxicity

induced by Aβ-stimulated microglia.

4. Discussion

Studies performed over the past few decades have increasingly indicated that

microglia-mediated neuroinflammatory responses are important in AD pathogenesis.

In the brain of patients with AD, deposition of Aβ elicits the formation of abundant

reactive microglia around senile plaques (Itagaki et al., 1989). Aβ serves as a

neuroinflammatory stimulus for microglia, and Aβ-dependent microglial activation

induces neuronal injury because of the secretion of various proinflammatory

molecules such as TNFα, IL-6, and IL-1β, reactive oxygen species, and reactive

nitrogen species (Agostinho et al., 2010). Both epidemiological and clinical data have

indicated the efficacy of anti-inflammatory therapies for delaying the progression and

for decreasing the symptomatic severity of AD (Zhou and Hu, 2013). These findings support the idea that inhibition of microglial inflammation is a potentially valuable therapeutic strategy for treating AD. The present study showed that microglial activation resulted in the synthesis and secretion of proinflammatory cytokines such as TNFα, IL-6, and IL-1β and that this response was associated with iNOS-derived nitrosative stress events. Specifically, TNFα was identified as the principal neurotoxic agent resulting from the proinflammatory transcriptional changes, which induced neuronal apoptosis after treatment with conditioned media from Aβ-stimulated microglia. In contrast, EGCG markedly suppressed Aβ-induced cytotoxicity by reducing reactive oxygen species-induced NF-κB activation and MAPK signaling, including JNK and p38 signaling. Particularly, EGCG increased the expression of Nrf2 and HO-1, which act as important endogenous antioxidants against free radical-induced proinflammatory effects in microglia (Park et al., 2013a). Hafner et al. (2013) have indicated that secretion of γ-enolase by microglia exerts protective effects on neuronal cells exposed to Aβ. Because EGCG promotes the expression of γ-enolase in human neuronal cells (Hossain et al., 2012), EGCG may prevent neurotoxicity through a similar mechanism. Our results provide a mechanistic explanation of how Aβ-stimulated microglia induce oxidative damage-associated neuronal apoptosis, suggesting that EGCG could be used as a neuroprotective agent for treating Aβ-induced neurotoxicity.

AD-associated inflammatory responses are mediated by microglial activation. The precise mechanisms underlying Aβ-mediated microglial activation are unclear. However, increasing evidence suggests that oxidative stress is strongly associated with microglial neuroinflammation. High concentrations of Aβ do not cause serious neuronal damage if microglia are absent in the brain of patients with AD (Giulian, 1999). Neuroinflammatory molecules released by activated microglia directly

promote neuronal toxicity and neurodegeneration (Surace and Block, 2012). Among

several factors that mediate neuroinflammation, TNFα is the principal mediator of a

cascade of cellular events that culminate into neuronal cell death. Many studies have

shown that TNFα exerts a deteriorating effect in AD pathology. For example,

APP/presenilin 1 transgenic mice treated with anti-TNFα monoclonal antibody show

reduced tau pathology and amyloid plaque deposits (Shi et al., 2011). Furthermore,

long-term inhibition of TNFα ameliorates neurodegeneration and memory impairment

in a $3 \times$ Tg mouse model of AD (Gabbita et al., 2012). Hensley et al. noted that

coincubation of EOC-20 murine microglia with TNFα and IL-6 significantly induced

NO_2 production and neuroinflammation (Hensley et al., 2003). Consistent with these

findings, our results showed that EGCG significantly suppressed TNFα and regulated

downstream signal transduction pathways in microglia by blocking NF-κB and

MAPK signaling and by partially exerting a protective effect against oxidative

stress-induced damage by eliciting endogenous antioxidant production. This

suggested that EGCG could be a useful intervention for treating microglia-induced

neuroinflammation and supported the practicability of targeting TNFα by EGCG as a

therapeutic strategy for treating Aβ-mediated neurotoxicity.

Accumulating preclinical evidence suggests that EGCG is a potent

neuroprotective agent for treating AD. However, transport of small molecules such as

EGCG through the blood–brain barrier can increase its bioavailability in the brain.

This has been confirmed by several studies that showed EGCG crosses the

blood–brain barrier and reaches the functional parts of the brain (Lin et al., 2007;

Singh et al., 2008). This suggests that EGCG exerts preventive or therapeutic effects

for protecting against Aβ-induced neurotoxicity. In addition, EGCG appears to be safe

and can be included in diet even at relatively high doses. Previous studies have shown

that up to 6 mg/kg of EGCG can be consumed without any adverse effects (Lee et al.,

2013; Rietveld and Wiseman, 2003). Therefore, EGCG can be used in further clinical trials without the risk of significant side effects. In summary, our present study showed that Aβ-stimulated microglia induced proinflammatory cytokine expression and secretion, whereas EGCG inhibited Aβ-induced overexpression of proinflammatory molecules, including IL-1β, IL-6, TNFα, and iNOS, in EOC 13.31 microglia. In addition, EGCG markedly suppressed Aβ-induced activation of MAPK signaling molecules such as JNK and p38 and protected neuro-2a cells against microglia-induced neurotoxicity involving TNFα-mediated neuronal death. The proposed scheme for the effect of EGCG on the suppression of Aβ-stimulated proinflammatory events in microglia is shown in Fig. 4E. Together, our findings provide a mechanistic explanation of how Aβ-stimulated microglia induce neuroinflammatory responses and suggest that EGCG has therapeutic potential for treating Aβ-induced neurotoxicity in patients with AD.

5. Conflict of interest statement

The authors declare there are no actual or potential conflicts of interest.

6. Acknowledgements

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Figure legends

Figure 1. EGCG inhibits Aβ-induced neuroinflammatory molecules in EOC 13.31

microglia. (A) Effects of EGCG on the viability of EOC 13.31 microglia. EOC 13.31

cells were treated with the indicated concentrations of EGCG in the presence or

absence of 10 μM Aβ for 24 h. Cell viability was assessed by performing the MTT

assay; results are expressed as mean ± S. E. M. of surviving cells compared to

untreated controls. (B) The mRNA levels of TNFα, IL-6, IL-1β, and iNOS were

measured by performing real-time RT-PCR; results are expressed as mean ± S. E. M.

Microglia were incubated with 100 ng/mL LPS as a positive control. (C)

Concentrations of TNFα, IL-6, and IL-1β in the culture media were measured using

an ELISA kit. All data are obtained from three independent experiments, and values

are expressed as mean ± S. E. M. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with untreated

groups, and $*P < 0.05$ and $**P < 0.01$ were compared with Aβ alone groups for

multiple comparisons by using Dunnett's post-hoc test.

Figure 2. EGCG suppresses Aβ-induced reactive oxygen species production in EOC

13.31 microglia. EOC 13.31 cells were treated with the indicated concentrations of EGCG and incubated with or without 10 μM Aβ. After 24 h of stimulation, the cells were incubated with DCFH-DA for an additional 0.5 h. Intracellular levels of reactive oxygen species were determined by using a fluorescence microscope (A) or flow cytometry (B). (C) Protein expressions of p-IκB-α, IκB-α, cytosolic p65, and nuclear p65 were determined by performing western blotting with specific antibodies. (D) EOC 13.31 microglia were transfected with an NF-κB luciferase reporter plasmid and treated with the indicated compounds for 24 h. Cell lysates were prepared, and luciferase activity was measured. (E) Immunoblotting showed that Nrf2 and HO-1 antioxidant pathways were suppressed after treatment with 10 μM Aβ for 24 h and that this inhibition was effectively reversed by EGCG. All data are obtained from three independent experiments, and values are expressed as mean ± S. E. M. *$P <$ 0.05 and **$P < 0.01$ were compared with Aβ alone groups for multiple comparisons with Dunnett's post-hoc test; scale bar = 20 μm.

Figure 3. EGCG inhibits Aβ-induced activation of MAPK signaling in EOC 13.31 microglia. EOC 13.31 cells were treated with different concentrations of EGCG and incubated with or without 10 μM Aβ for 24 h. Protein expression of phosphorylated JNK and JNK (A), phosphorylated p38 and p38 (B), and phosphorylated ERK and ERK (C) was determined by performing western blotting with specific antibodies. Relative band intensities were transformed using a histogram. (D) Effects of MAPK inhibitors on Aβ-induced upregulation of TNFα mRNA expression. EOC 13.31 microglia were stimulated with 10 μM Aβ and treated with the indicated concentrations of specific kinase inhibitors (SP600125 for JNK, SB203580 for p38, and PD98059 for ERK1/2) for 24 h. The mRNA levels of TNFα were measured by performing qPCR; the results are expressed as mean ± S. E. M. The relative

expression of proteins was quantified densitometrically by using Quantity One (Bio-Rad) and was calculated using non-phosphorylated reference bands. All data are obtained from three independent experiments. *P < 0.05 and **P < 0.01 were compared with Aβ alone groups for multiple comparisons by using the Dunnett's post-hoc test.

Figure 4. Protective effects of conditioned medium (CM) from EGCG-treated EOC 13.31 microglia against Aβ-induced neurotoxicity in neuro-2a neuronal cells. (A) Effects of EGCG on the viability of neuro-2a cells. Neuro-2a cells were treated with the indicated concentrations of EGCG in the presence or absence of 10 μM Aβ for 24 h. (B) Neuro-2a cells were stimulated with 10 μM Aβ in the presence or absence of 10 μM EGCG or 100 ng/mL anti-mouse TNFα antibody for 24 h and assessed by performing TUNEL or MTT assay. (C) EOC 13.31 microglia were stimulated with 10 μM Aβ in the presence or absence of 10 μM EGCG or 100 ng/mL anti-mouse TNFα antibody for 24 h. Next, the CM were transferred to neuro-2a cells. After 24 h, neuro-2a cells were assessed by performing TUNEL or MTT assay. (D) Treated neuro-2a cells were lysed after stimulation, and nitrotyrosine-containing proteins were immunoprecipitated and resolved by performing SDS-PAGE on an 8% gel and western blotting by using anti-iNOS and anti-nitrotyrosine antibodies. (E) A proposed scheme for the protective effects of EGCG against Aβ-induced microglia-mediated neuroinflammation. All data are obtained from three independent experiments, and values are expressed as mean ± S. E. M. *P < 0.05 and **P < 0.01 were compared with Aβ alone groups for multiple comparisons by using Dunnett's post-hoc test; N. S., no significant difference.

Responses to the Reviewer's comments (EJP-41694 R2)

By your suggestion, our manuscript has been carefully corrected for the English grammar and style by a professional scientific English editing service. We now make our substantial revision with point-by-point statement of responses to the reviewer's comments.

REVIEWERS' COMMENTS:

Reviewer #1:

The manuscript seems to be improved. however, I am not so sure if the English editing has been done carefully. For example, the citations seem to be full of mistakes. The abbreviations of the journals were not written correctly according to the author guidelines. Moreover, the authors did not use IUPAC guidelines in the use of the units in their figures. For example ng/ml should be ng/mL in the text and also in Fig. 1. In Figure 1A, "Concentrations of EGCG" should be [EGCG] (uM). Thus, I have to recommend a minor revision of the manuscript with more careful attention to English editing.

Answer: Thank you for correcting our mistakes. Now all the figures are modified by IUPAC guidelines. In addition, our manuscript has been corrected for the English grammar and style by a professional scientific English editing service. We hope this results in a paper that is easier to read and understand.

Comment from the Editor: this paper must be carefully reviewed for language by a native English speaking collaborator.

Answer: By your suggestion, the manuscript is carefully reviewed for language by a native speaking English service.

Checklist for style revision:
- In the title capitalize only the first letter of the first word.

Answer: As requested, only the first letter of the first word is capitalized.

- Do not use a justified layout, align only left.

Answer: As requested, the text is aligned only left in this version.

- Do not use ROS as abbreviation, write in full throughout the manuscript.

Answer: As suggested, all the abbreviation of "ROS" are replaced by the full term "reactive oxygen species" throughout the manuscript.

- Use S.E.M. (not SEM).

Answer: As requested, all the words "SEM" are corrected to "S.E.M.".

- To indicate statistical significance use the uppercase P instead of p.

Answer: As requested, uppercase P is applied to all the statistical significance symbol.
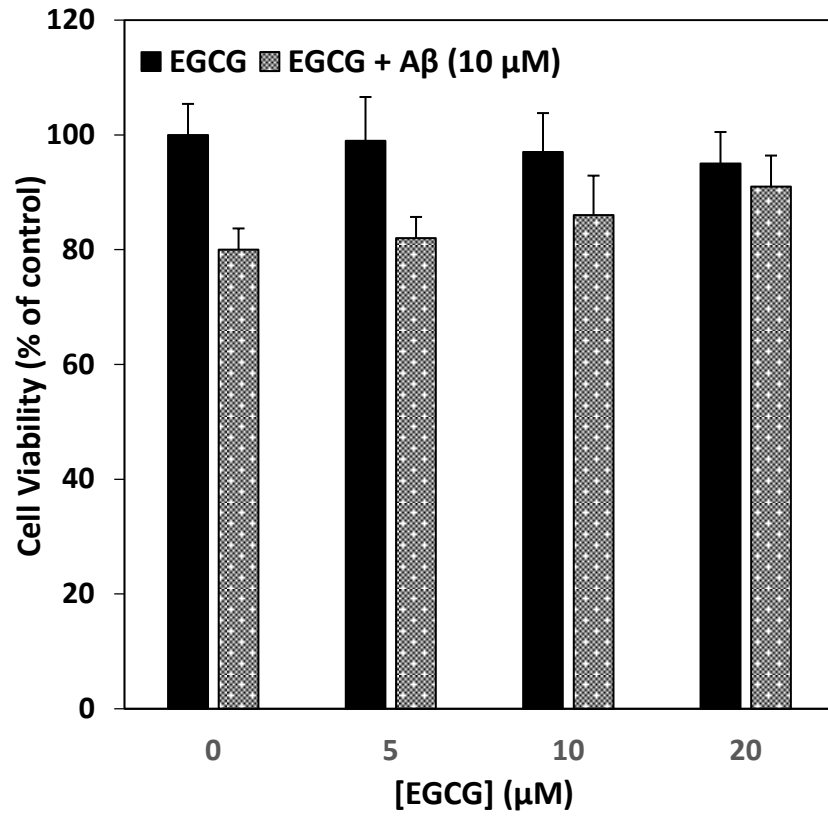
Dear Dr. F.P. Nijkamp, Editor in chief, *European Journal of Pharmacology*

We thanks a lot to receive your review for our manuscript (EJP-41694R1) entitled "Epigallocatechin gallate attenuates amyloid β-induced inflammation and neurotoxicity in EOC 13.31 microglia" on Sep 11. You have kindly indicated that our manuscript could be resubmitted after alter the manuscript according to the comments made by reviewers. We now present our substantial revision. In addition, our manuscript is carefully reviewed for language by a native speaking English service, and hope this revision is easier to read and understand. We would like to accept any further suggestions that could improve our works. Thank you once again for your kindly review of our manuscript.

Sincerely,

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Phone: +886-4-2473-0022, ext. 12405; Fax: +886-4-2472-3229
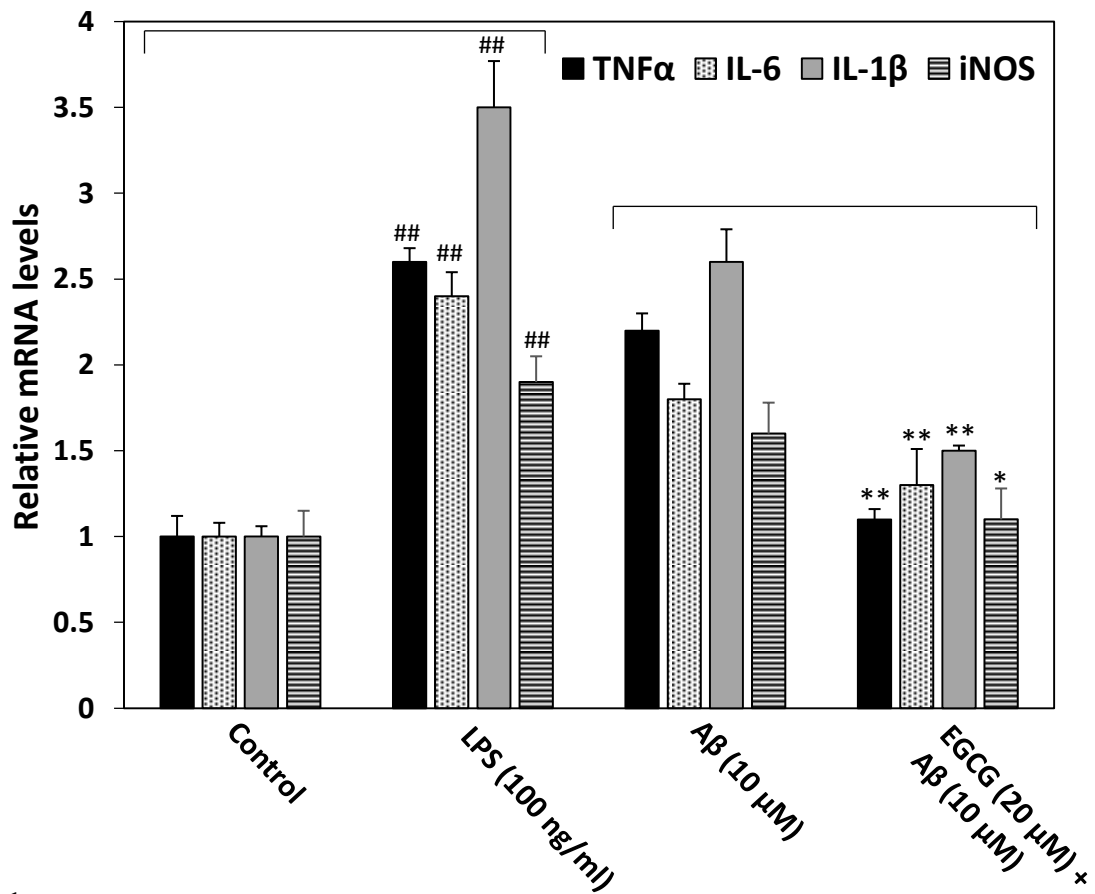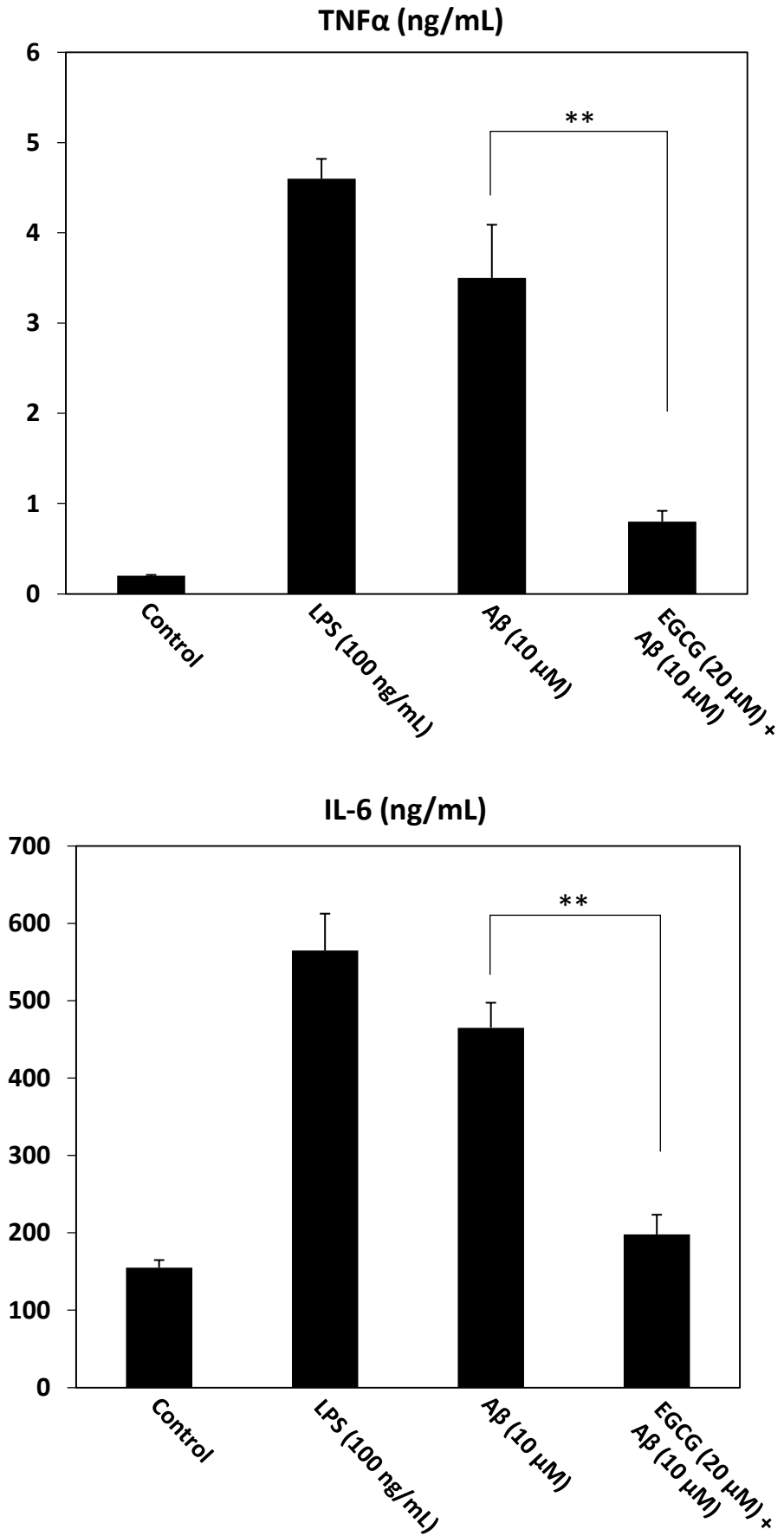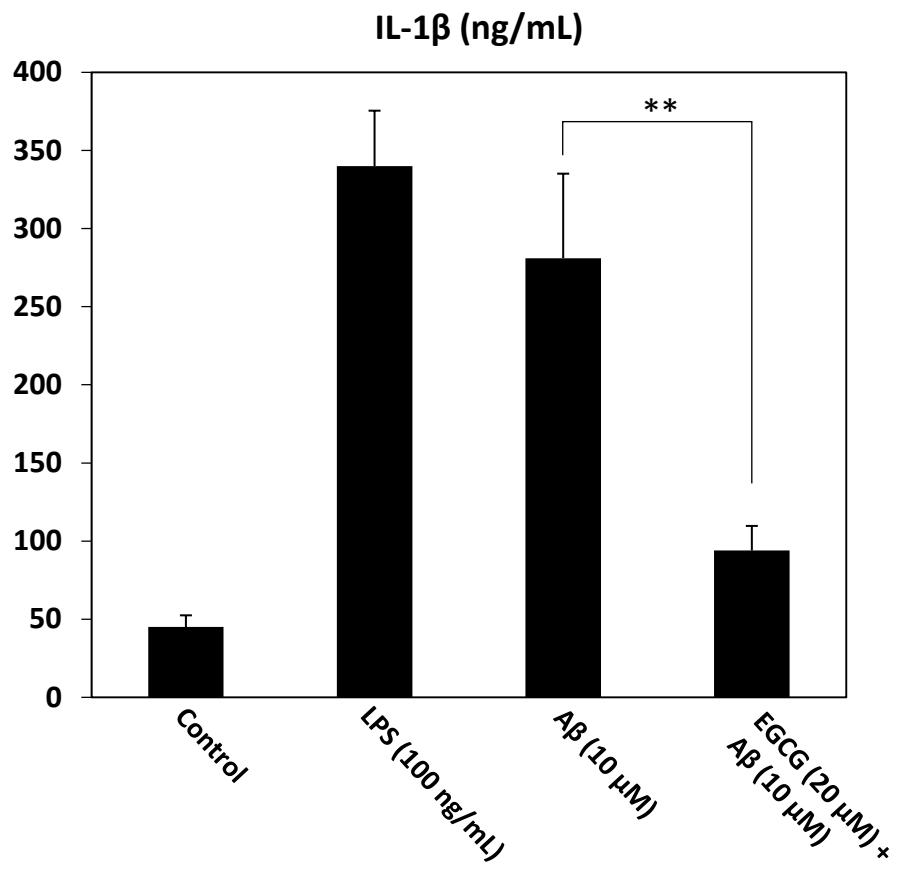E-Mail: dll@csmu.edu.tw

A.

B.

Figure 1
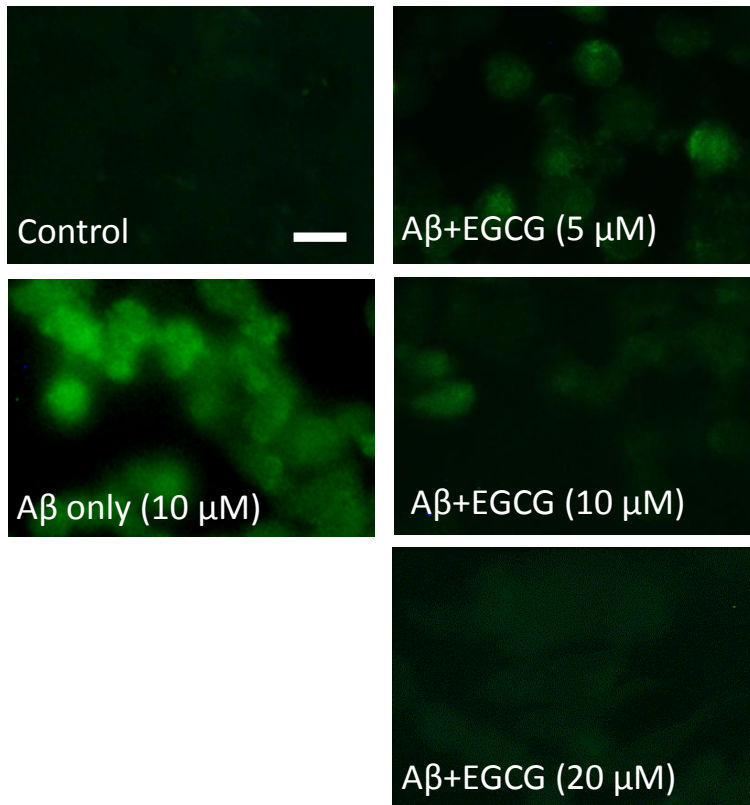
C.

TNFα (ng/mL)

IL-6 (ng/mL)

Figure 1

IL-1β (ng/mL)

Figure 1 (continued)

A.

B.

Figure 2

C.

		[Aβ] (10μM)		
[EGCG] (μM)	-	-	**5**	**10**	**20**

p-IκB-α

1.0±0.15 2.1±0.23 1.4±0.25 1.1±0.08 0.2±0.06

IκB-α

1.0±0.06 0.5±0.10 0.6±0.18 0.9±0.06 1.0±0.14

Cytosolic p65

1.0±0.08 0.5±0.09 0.6±0.10 0.7±0.12 0.9±0.13

Nuclear p65

1.0±0.08 2.8±0.39 1.6±0.21 1.2±0.08 0.9±0.06

D.

[Aβ] (10 μM)	-	+	+	+	+	+
[EGCG] (μM)	-	-	5	10	20	-
[NAc] (100 μM)	-	-	-	-	-	+

Figure 2

E.

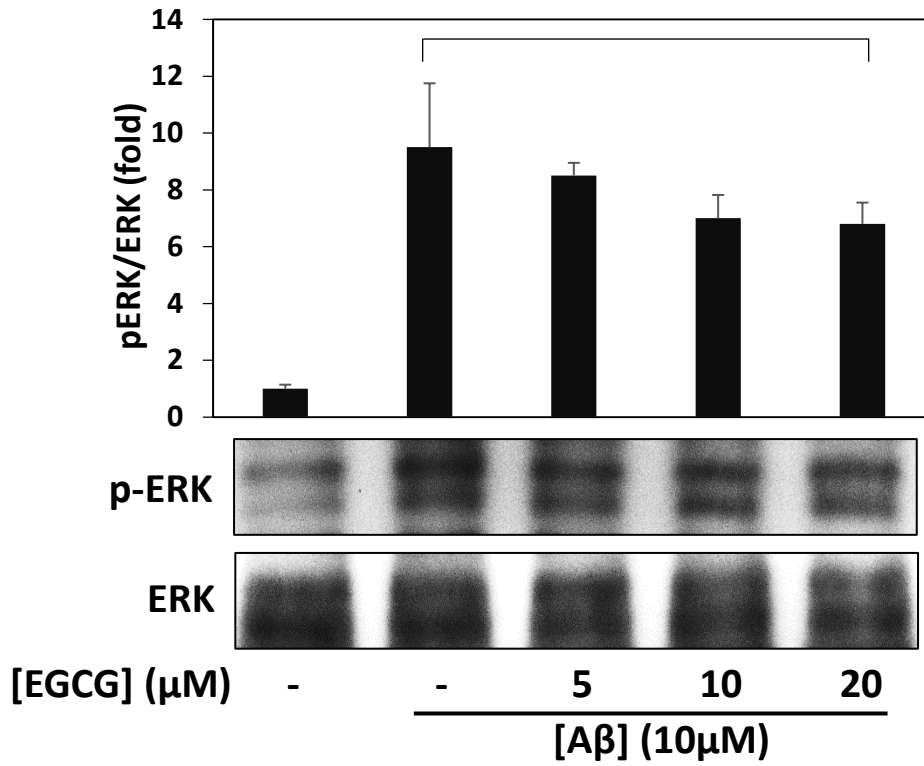|	without Aβ		with Aβ	
[EGCG] (10 μM)	-	+	-	+
Nrf2	1.0±0.10	1.1±0.15	0.6±0.08	0.8±0.04
HO-1	1.0±0.05	1.3±0.20	0.5±0.09	1.1±0.14
β-actin				
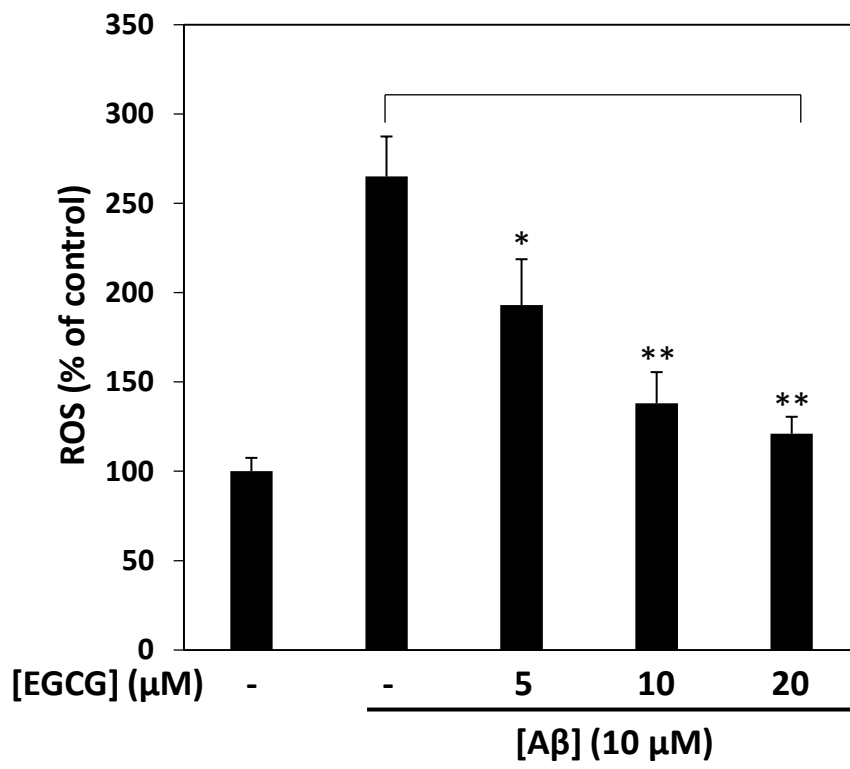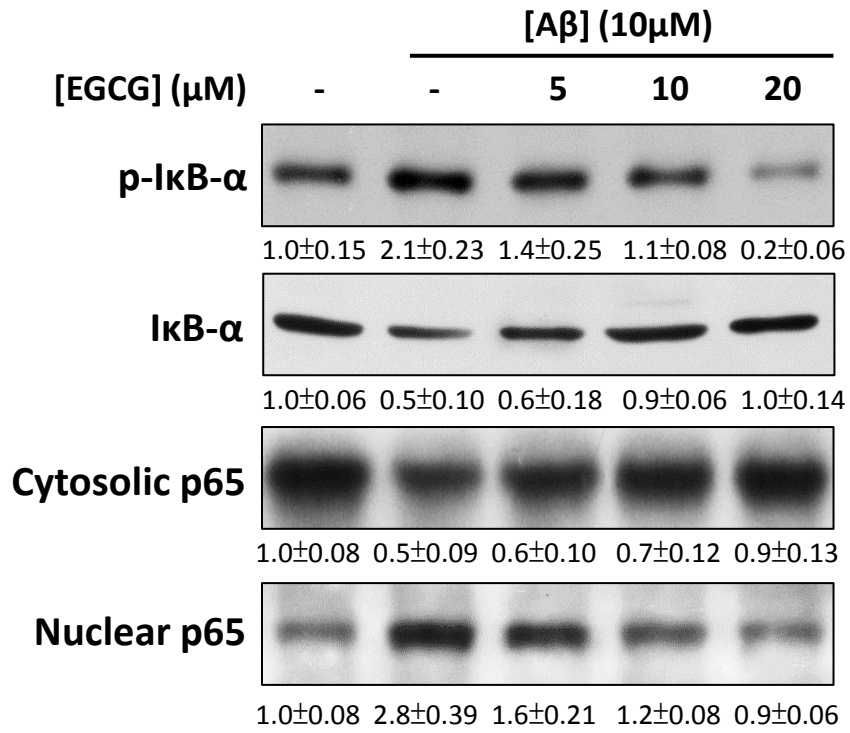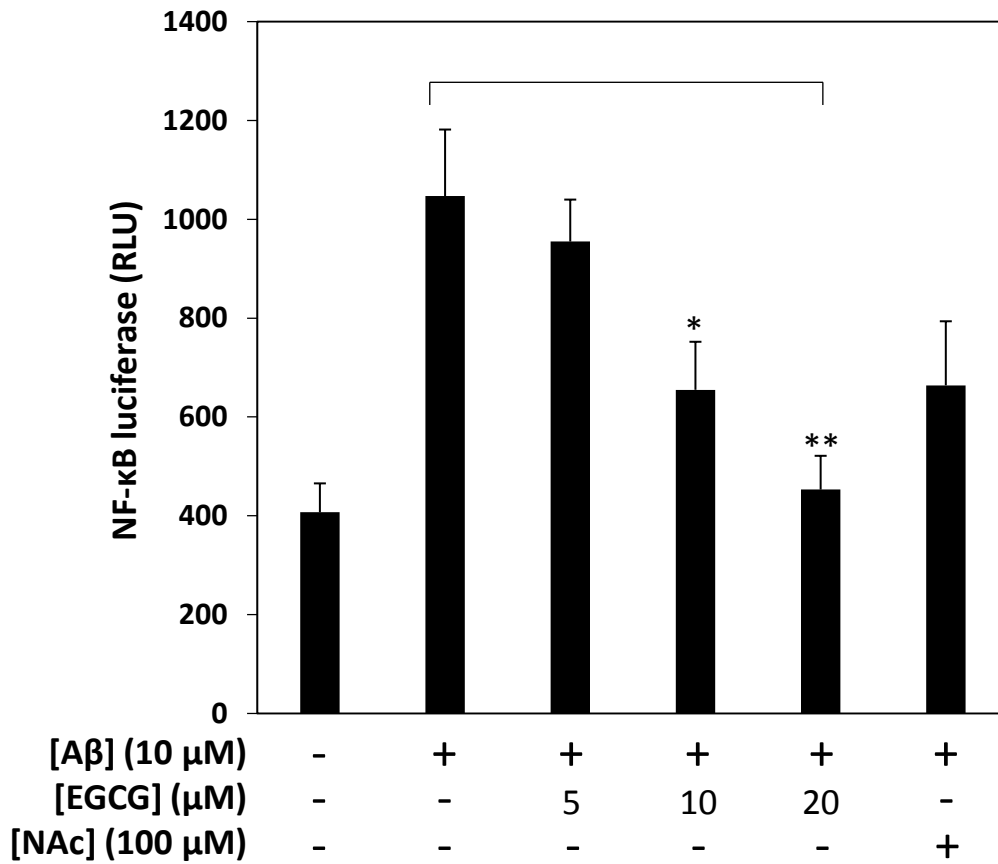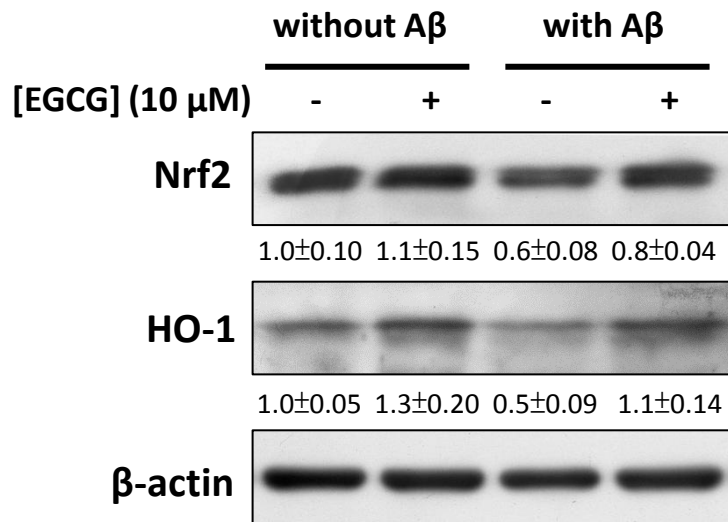
Figure 2

A.
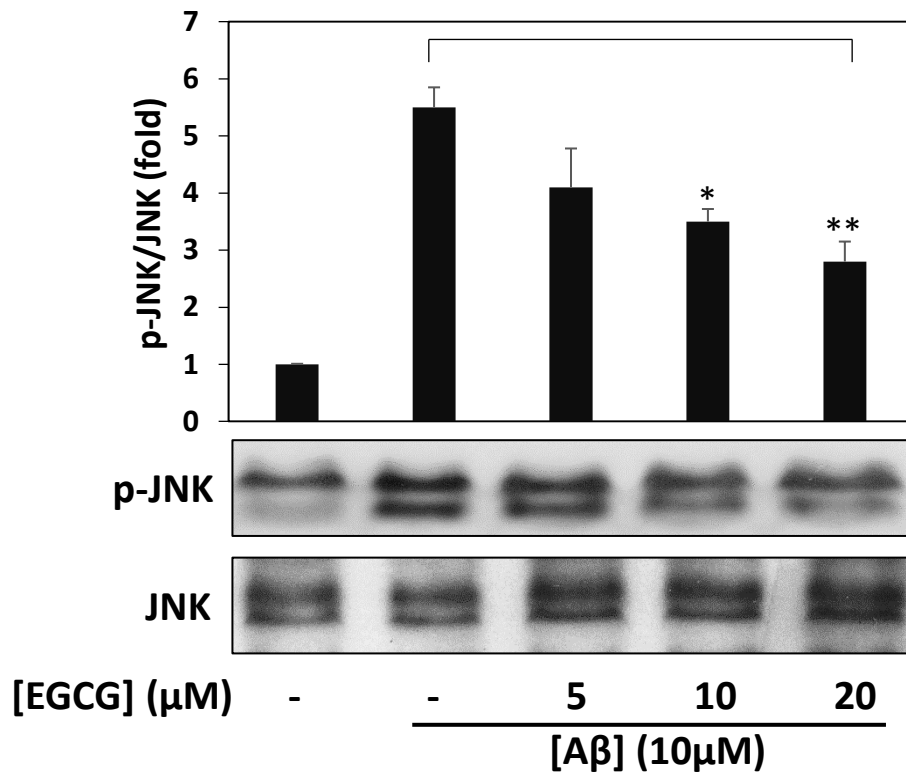
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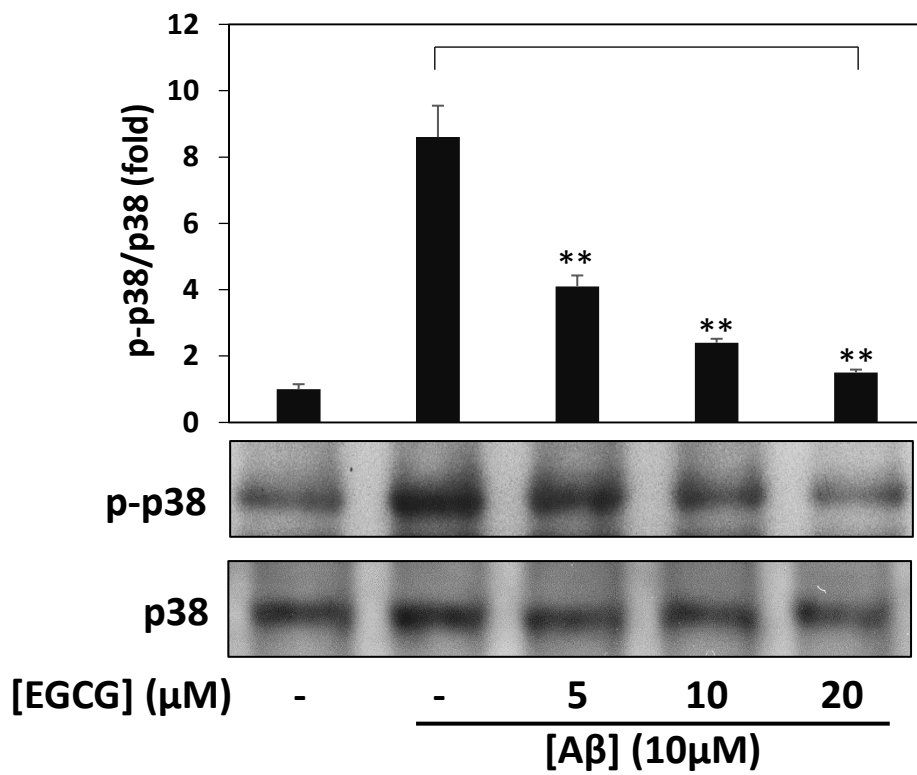
Figure 3

C.

D.

Figure 3

A.

Figure 4

B.

TUNEL (witout CM)

MTT (without CM)

	Aβ	EGCG	anti-TNFα Ab
	−	−	−
	+	−	−
	+	+	−
	+	−	+

C.

TUNEL (with CM)

MTT (with CM)

	Aβ	EGCG	anti-TNFα Ab
	−	−	−
	+	−	−
	+	+	−
	+	−	+

Figure 4

D.

EOC 13.31

	No CM	CM only	CM (after treated with EGCG)
Aβ	-	+	+
EGCG	-	-	+

Neuro2A

nitro-Tyr

260 kDa
140 kDa
100 kDa
70 kDa
50 kDa
35 kDa

iNOS

1.0±0.12   2.2±0.36   1.1±0.08

β-actin
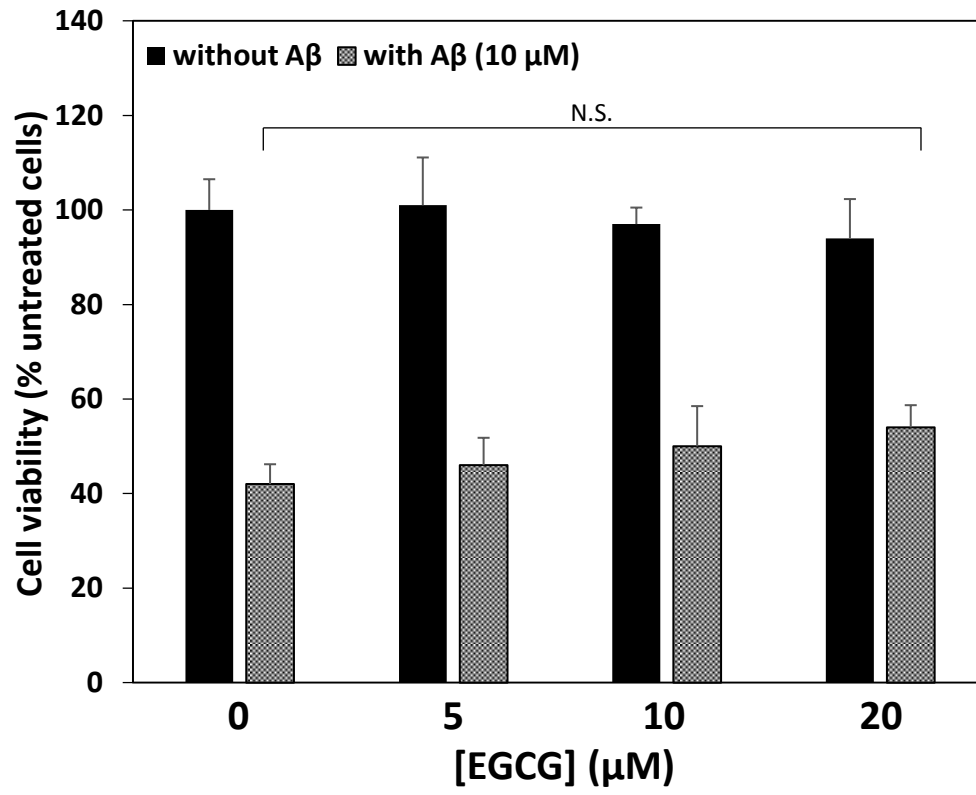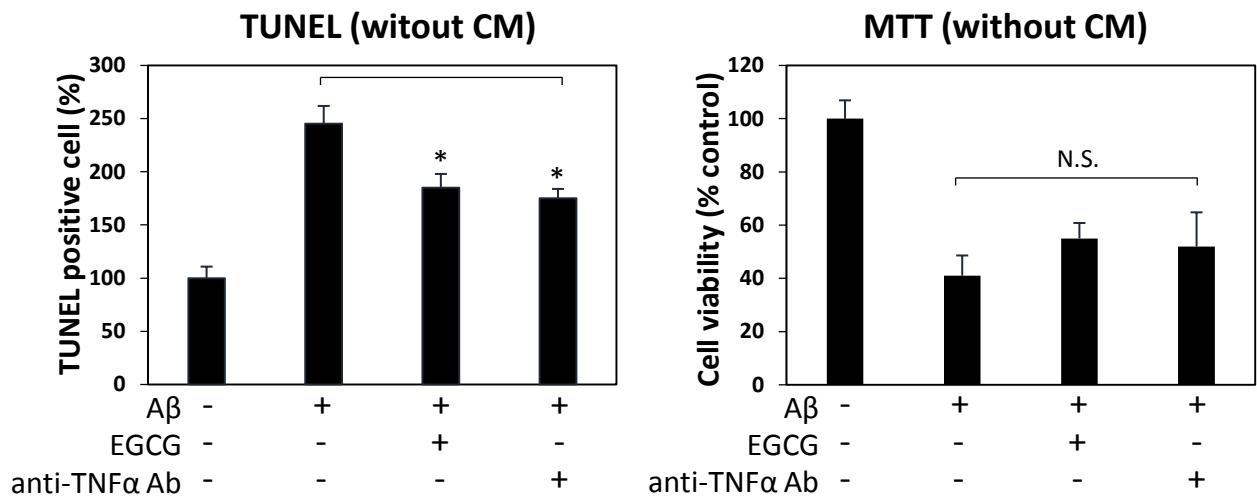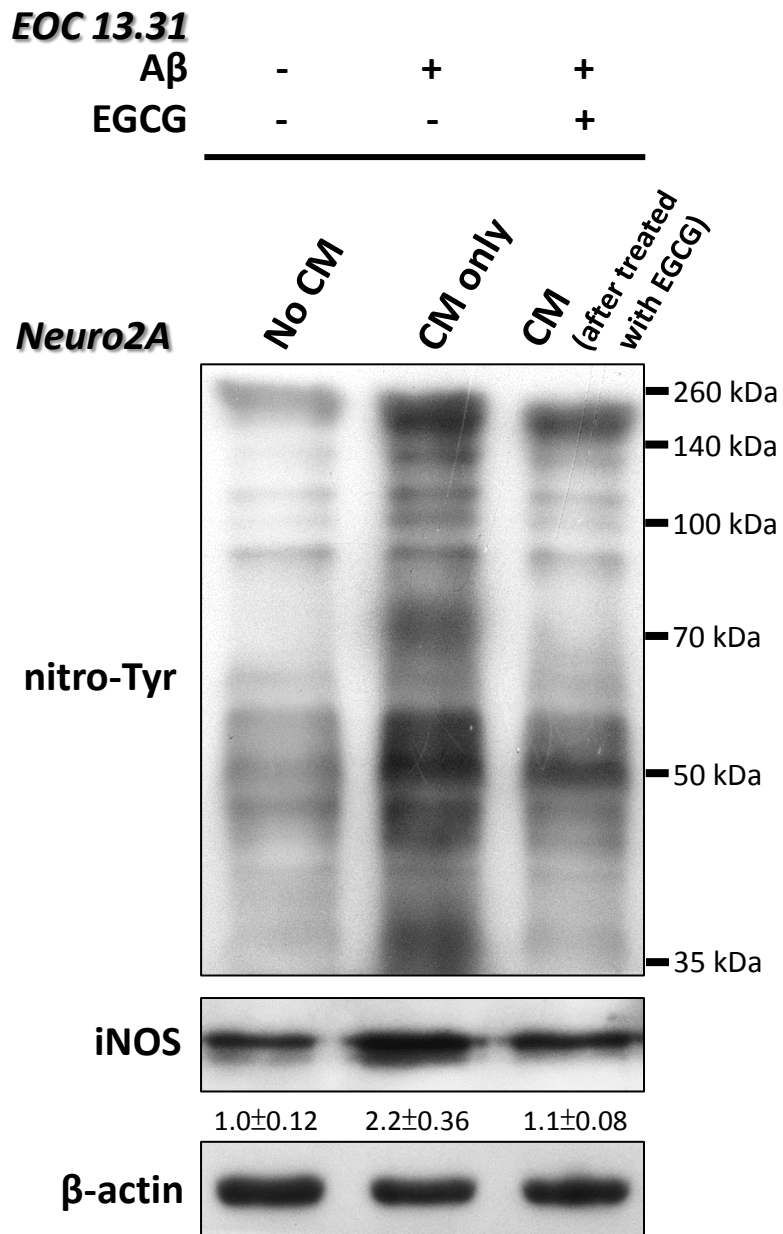
Figure 4

E.
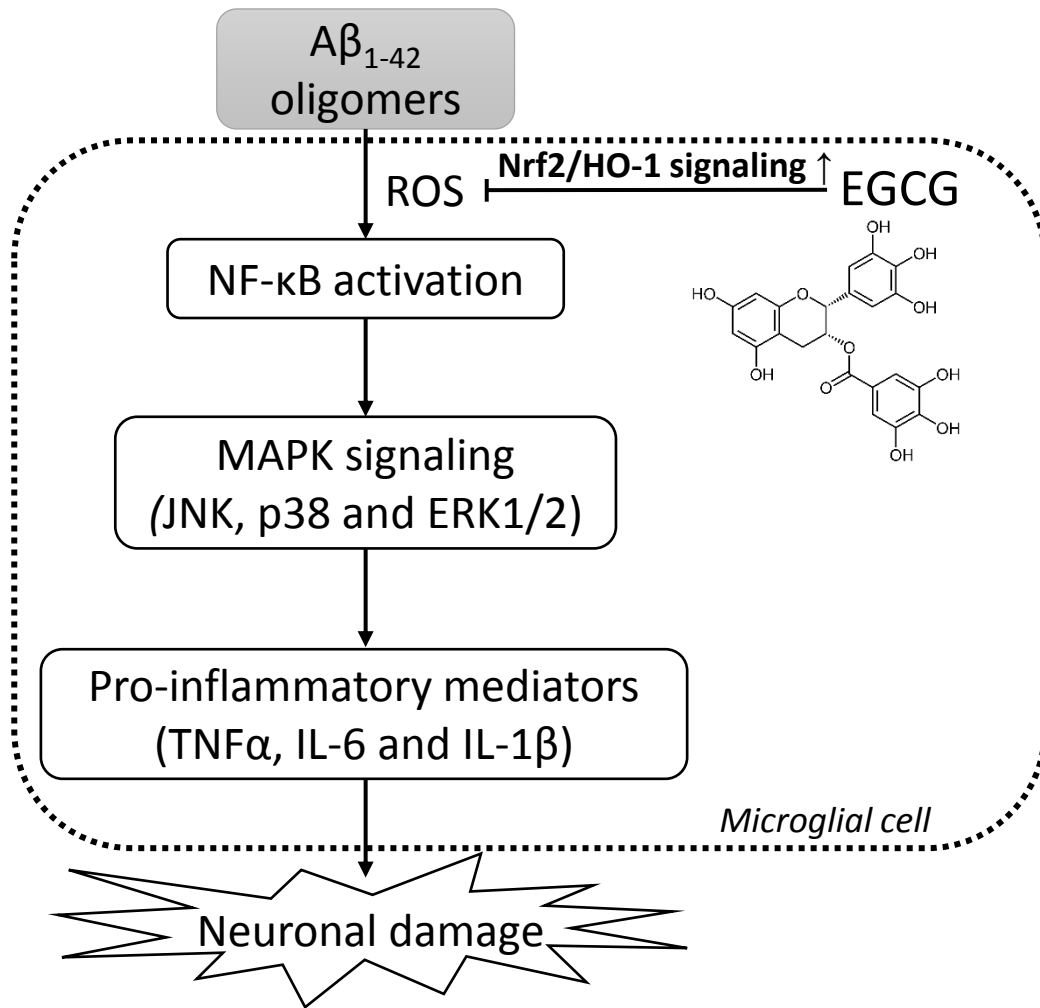
Figure 4

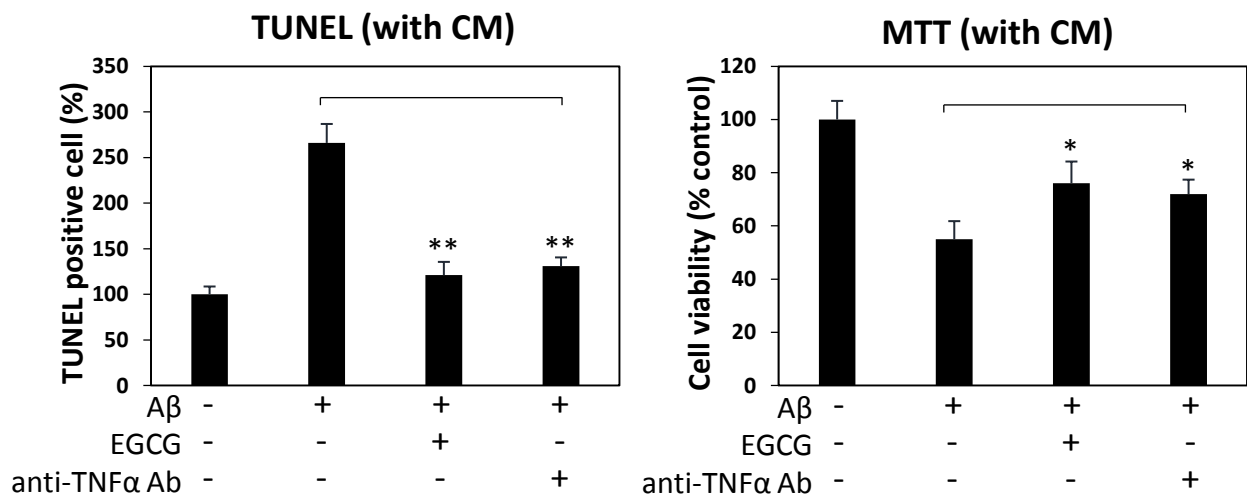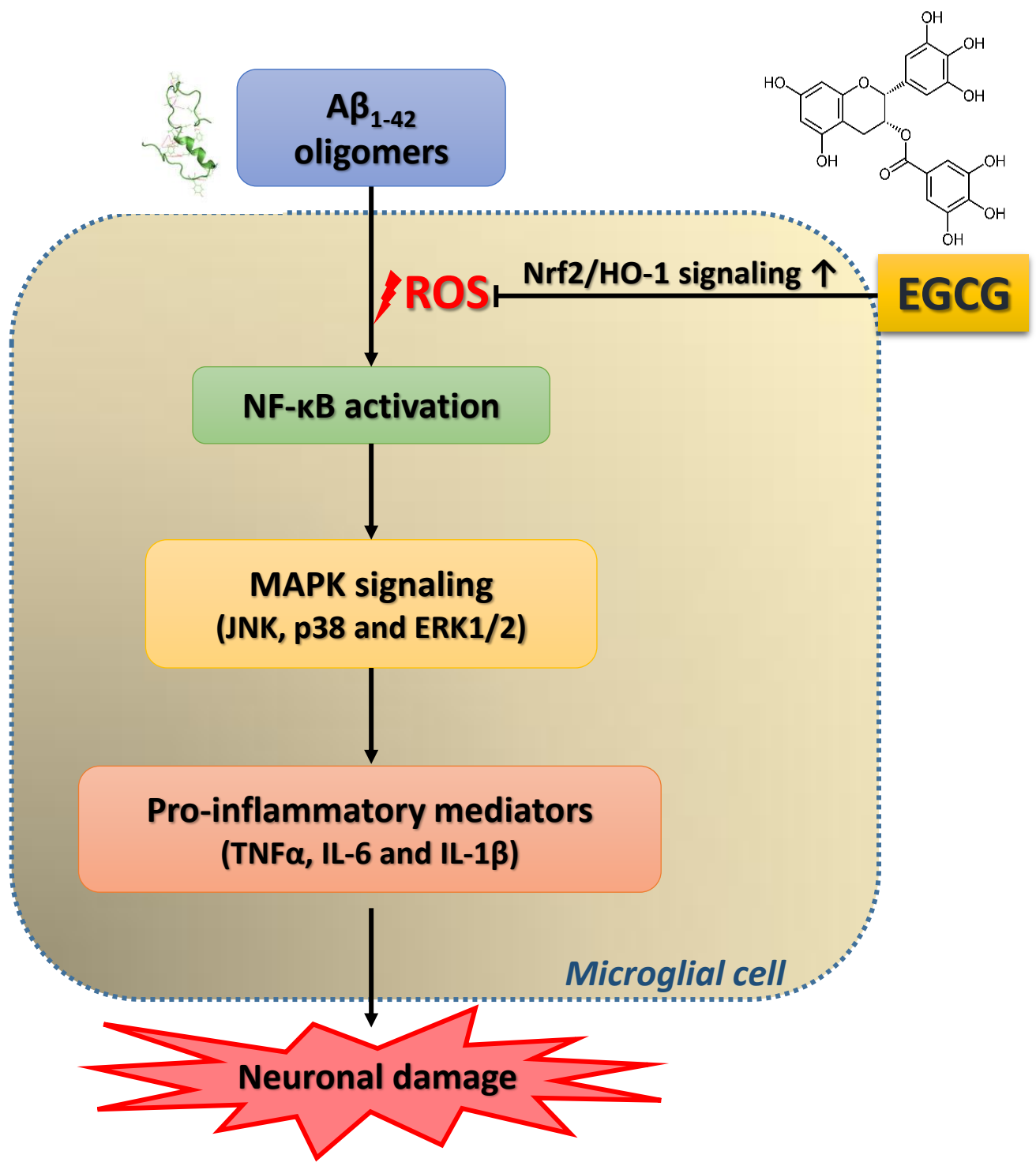**Supplementary material for online publication only**

國科會補助專題研究計畫

出席國際學術會議心得報告

日期：105 年 10 月 31 日

計畫編號	NSC　101 － 2320 － B － 040 － 015 － MY3		
計畫名稱	第一型類胰島素生長因子訊息途徑保護神經細胞對抗乙型類澱粉蛋白毒性之機轉研究		
出國人員姓名	林志立	服務機構及職稱	中山醫學大學醫學研究所副教授
會議時間	105 年 7 月 4 日至 105 年 7 月 9 日	會議地點	德國 Berlin
會議名稱	(中文) 第 40 屆歐洲生化年會 (英文) 40th Congress of the Federation of the European Biochemical (FEBS)		
發表題目	(中文) 誘導 Nanog 活性產生對抗乙型類澱粉蛋白毒性之神經保護作用 (英文) Induction of Nanog displays protective effects against amyloid β (Aβ)-induced cytotoxicity		

一、參加會議經過

　　FEBS (The Federation of European Biochemical Societies)為歐洲地區老牌的生化學定期會議，也是歐洲影響力最大的生化學盛會。此會議每年舉辦一次，今年選擇在德國的柏林舉辦。根據大會公告今年有將近 800 篇的海報被發表，2000 位以上註冊的會員參與本次會議，關於此次會議的參加心得報告，以下將就與會活動參與的內容簡要說明：本次 FEBS 討論的主題涵蓋所有生化學相關議題，今年重點的議題包括 Mechanisms of Gene Expression、Membranes, Receptors & Bioenergetics、Structural Biology and Biophysics、Systems Biology, Bioinformatics & Theoretical Biology、Molecular Neuroscience、以及 From Chemical Biology to Molecular Medicine 等議題。本年度的開場演講請來 Heidelberg 大學的 Dr. Bernd Pulverer，講題是關於投稿生化學相關 SCI 期刊今後關切各議題的趨勢，Dr. Pulverer 是著名雜誌 The EMBO Journal 的總主編，以其雜誌廣泛收到各方面稿件的經驗，深入淺出分析未來研究熱點的大趨勢。個人的的海報論文被大會安排至 7 月 4 號下午至 7 月 6 日上午於編號 P22-011 的位置展示，並訂於 7 月 5 日 02:15 PM - 03:00 PM 擔任解說與答辯。

二、與會心得

本研討會開會的會場在 Estrel Berlin – Hotel & Convention Center 舉行，Estrel Berlin Hotel 位於柏林東側郊區，屬舊蘇聯東柏林的屬地，雖圍牆已倒塌三十年，但相對建設仍較西柏林落後，但另一方面來看建築物也比較不會那麼擁擠，周邊視野開闊舒暢。。而本次大會是在北側的會展中心進行，會場裡除了演講大廳外還有 6 個口頭發表場地及兩個大型海報發表場地。在每日 Plenary Lecture 大型場地至少有數百個個以上的座位供所有與會人士參與，而在其後的 Symposia 與 Special Lecture 中則每場次均有約 100 人左右的專家學者參與，但比較好奇的是雖 FEBS 應屬於大型會議，但每場次參加的人員較過去經驗(e.g. FENS、AAIC)而言相對較不踴躍，廠商參與的意願也明顯減少，這是蠻令我訝異的一件事。雖然如此，但本次會議過程成有幸遇到 UT (University of Texas)的 Dr. George Perry，其對於我們此次的研究非常感到興趣，似乎是特地來找我們的 poster 並針對某些議題進行討論。在討論的過程中我才發現他便是老牌雜誌 JAD (Journal of Alzheimer's Disease)的總主編，討論結束後他也很誠懇的當面向我邀稿，並在數天後再發一次 Email 給我正式邀稿函。目前本 poster 的結論也已依其邀請於 9 月底投稿到 JAD，並經過高效率的 reviewing 程序完成一審的動作，目前已經正在

進行 minor revision。

三、發表論文摘要

Abstract Preview - Step 3/4
- print version -

Topic: 18 Degeneration and ageing of the nervous system

Title: Induction of Nanog displays protective effects against amyloid β (Aβ)-induced cytotoxicit

Author(s): Lin C.-L.[1], Kim H.G.[1], Li H.-H.[1], Huang W.-N.[1], Huang C.-N.[1], Laboratory of Neuroscience and Endocrinology

Institute(s): [1]*Chung Shan Medical University, Institute of Medicine, Taichung, Taiwan, Republic of China*

Text: Alzheimer´s disease (AD) is the most common neurodegenerative disorder characterized by amyloid β (Aβ) deposition in the brain. Currently, AD therapies can only alleviate symptoms rathe than Aβ-induced neurodegeneration, thus providing no pathway to overcome Aβ toxicity. Previou studies have demonstrated that Nanog, a homeodomain-bearing protein required for maintenance of pluripotency, may be linked to the pathogenesis of AD. However, the exact mechanisms underlying Nanog´s contribution to AD are still unclear. In the present study, we evaluated the protective pathways by which Nanog protects against Aβ-induced cytotoxicity. Our results indicate that Nanog overexpression can counteract oxidative damage by neutralizing excessive ROS, thus contributing to the alleviation of Aβ-induced neurotoxicity. In addition, Aβ induced the loss of mitochondrial membrane potential and activation of caspase 3 and PARP, whereas overexpressed Nanog significantly attenuated these forms of deterioration. This protective effect may be due to the activation of AMP-activated protein kinase (AMPK) in a sirtui 1 (Sirt1)-dependent pathway by shifting endogenous reactive oxygen species (ROS) detoxification responses away from cell death and toward survival. We expect our results can provide the basis for molecular mechanisms involved in the pathogenesis of brain Nanog signaling and AD. Accordingly, stimulation of Nanog signaling by targeting Nanog may lead to novel therapeutic strategies by slowing or halting AD progression in future.

FEBS Bursary: I will **NOT** apply for a FEBS bursary

四、建議

本次會議的流暢性以及深入性皆有相當的專業水準，與國內大型會議比較起來，但還是有一些尚可進步之處，包括：

（1）雖然本次會議參加人數十分眾多，但報到流程、會場動線、各處標幟等皆十分完善與醒目，註冊等待時間也相當合理。

（2）會議場址稍嫌偏遠，周邊行人不多會令神經較為敏感的與會者有治安上的疑慮，唯德國的治安相當不錯，這層顧慮應屬杞人憂天，但還是建議能考慮大眾運輸較為方便之處，對於眾多的國際與會者而言會較為友善。

以上各點為我觀察到國內大型研討會不同之處。。最後，本人由衷地感謝科技部能補助個人參加本次論文發表的費用，此行的收穫豐盛，除第一次被雜誌社主編當面邀稿外，確實可對個人未來的研究有極大的影響與助益。

五、攜回資料名稱及內容

1. 大會會議手冊(電子檔)

2. 大會發表論文摘要(電子檔)

3. 藥廠及儀器商所提供之相關資料

六、其他

參與大會之照片

FEBS 2015 大會入口

海報論文展覽場所一隅

海報論文展覽場入口

科技部補助計畫衍生研發成果推廣資料表

日期：2015/10/30

科技部補助計畫	計畫名稱： 第一型類胰島素生長因子訊息途徑保護神經細胞對抗乙型類澱粉蛋白毒性之機轉研究		
	計畫主持人： 林志立		
	計畫編號： 101-2320-B-040-015-MY3　　　學門領域： 保健營養		
研發成果名稱	（中文）富氫水生成裝置		
	（英文）Gereration device of hydrogen-rich water		
成果歸屬機構	中山醫學大學	發明人 （創作人）	林志立, 呂鋒洲, 黃玟儂
技術說明	（中文）水是重要的生存必需品，每天人體都需要攝取足夠的水，方能達到人體代謝與正常運作的功能。功能水是目前一個發展方向，因為許多研究開始發現水也具有生理作用，能調節氧化壓力，改善疾病，例如：電解水。氫分子是一種具有前瞻性的抗氧化劑，於2007年發現氫氣具有掃除自由基的能力，具有良好的抗氧化能力。許多研究因此都開始發現其具有對抗氧化壓力造成之疾病的能力。氫分子是不具有毒性，非特殊催化或熱力條件下活性低，是一種鈍性氣體。富氫水（Hydrogen-rich water；HRW）是將氫分子溶於水之中，具有改善氧化壓力造成疾病的效果。目前有63種疾病動物模式、細胞模式，或臨床實驗證明其正向效果。本研究將開發HRW機，並加以探討其對抗阿茲海默氏症（Alzheimer's disease；AD）的能力。AD是一種高盛行率的漸進是腦部退化性疾病，是一種最常見的失智症，主要病理特徵有老年性斑塊堆積、神經纖維纏結與神經元流逝。老年性斑塊是由類澱粉胜肽（Amyloid-beta peptide；Aβ）過度堆積造成，Aβ會自由基使得氧化壓力產生，引發神經過度發炎、造成神經元損傷。AD的發病期漫長，對於患者本身、社會付出與家庭都是嚴重的負擔。HRW的研發主要目的在於由日常生活中，以保健的角度消除自由基，對抗AD的發生。		
	（英文）Amyloid β (Aβ) peptides are identified in cause of neurodegenerative diseases such as Alzheimer's disease (AD). Previous evidence suggests Aβ-induced neurotoxicity is linked to the stimulation of reactive oxygen species (ROS) production. The accumulation of Aβ-induced ROS leads to increased mitochondrial dysfunction and triggers apoptotic cell death. This suggests antioxidant therapies may be beneficial for preventing ROS-related diseases such as AD. Recently, hydrogen-rich water (HRW) has been proven effective in treating oxidative stress-induced disorders because of its ROS-scavenging abilities. However, the precise molecular mechanisms whereby HRW prevents neuronal death are still unclear. In the present study, we evaluated the putative pathways by which HRW protects against Aβ-induced cytotoxicity. Taken together, our findings suggest that HRW may have potential therapeutic value to inhibit Aβ-induced neurotoxicity.		
產業別	研究發展服務業, 藥品檢驗業, 其他專業、科學及技術服務		
技術/產品應用範圍	神經保護、抗老化		
技術移轉可行性及 預期效益	新型保健食品		

註：本項研發成果若尚未申請專利，請勿揭露可申請專利之主要內容。

101年度專題研究計畫研究成果彙整表

| 計畫主持人：林志立 | 計畫編號：101-2320-B-040-015-MY3 |

計畫名稱：第一型類胰島素生長因子訊息途徑保護神經細胞對抗乙型類澱粉蛋白毒性之機轉研究

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數（含實際已達成數）	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	6	11	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	1	1	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	4	5	100%	人次	
		博士生	1	2	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	3	10	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	3	5	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	1	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
其他成果（無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）		1. AMPK神經保護部分受邀至日本演講 2. Lambert Academic Publishing邀請撰寫教科書 3. 國際合作(俄羅斯、韓國)，並有一名韓國研究生到實驗室參與神經幹細胞研究					

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

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1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　　　□實驗失敗
   　　　□因故實驗中斷
   　　　□其他原因
   說明：

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2. 研究成果在學術期刊發表或申請專利等情形：
   論文：■已發表 □未發表之文稿 □撰寫中 □無
   專利：■已獲得 □申請中 □無
   技轉：□已技轉 □洽談中 ■無
   其他：（以100字為限）

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3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以500字為限）
   根據我們的實驗結果獲致以下的結論：
   1. 累積於腦部的Aβ其誘發之細胞毒性其機制可能是經由阻礙神經細胞的insulin signaling而導致神經退化，本研究易結果證實透過減緩brain insulin resistance可有效抑制Aβ所誘發之神經毒性。
   2. 我們的實驗釐清了insulin signaling與Aβ神經毒性之間的關係，特別是首先發現Nanog這個幹細胞相關蛋白在此機制中所扮演的角色，並證實可透過調控insulin signaling來影響細胞內Nanog表現，顯示將其應用於生物標記作為輔助AD臨床診斷的可能性。
   3. 在T2DM治療上，metformin與TZD是最被廣泛使用的第一線藥物，然而其對AD治療效果的分子機轉則有待實證。本研究結果證實透過改善insulin resistance及活化AMPK這兩種方式，都能幫助神經細胞對抗Aβ傷害，此結論將可提供部分anti-diabetic藥物在未來用於合併治療T2DM及AD的概念。
   4. 雖然幹細胞療法是治療人類神經病變的新遠景之一，但在胚胎幹細胞在使用及取得方面卻有其難以逾越的法律與倫理上的問題。而我們首先嘗試利用iPS方式，測試其應用在對抗Aβ神經毒性的可能潛力，預期這種neuronal iPS將可發揮幹細胞的生物特性，配合受損神經組織微環境的變化，動態地增進各種神經保護因子的旁分泌(paracrine)作用，同時並抑制膠細胞的過度活化及免疫反應，因此得以讓已受Aβ損傷的神經元有機會修復重生。
   5. 已知Aβ具有抑制autophagy的能力，而AMPK、Sirt1、PGC1α及

autophagy都是跟老化高度相關的蛋白，本研究也發現insulin signaling與這些老化相關蛋白之間的互動關係，今後並可能因此開發出全新思維的AD治療策略。