Distinguishing the Effect of Aging and SIRT1 Manipulation on AD progression with SIRT1 and APP Temporally Regulated Mice Models

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Abstract

Alzheimer's (AD) is a neurodegenerative disease closely linked to aging. However, mouse models with early onset AD are widely utilized, which may cause data deviations since AD normally occurs in aged subjects. In this work, we devised transgenic mouse models with SIRT1, Aβ42 regulated at the first month after birth and 19th months old via Tet-Off/CREer-LoxP systems. Aging/AD/SIRT1-interacted biomarkers were tracked throughout life. Based on the models, the effects of SIRT1, APP, and aging on AD progression are differentiated through temporal manipulation. The possible results of equal or more significant effects of SIRT1 deficiency on AD progression compared to aging hint the presence of pathology distinctly related to AD, which might lie in induced increased neuron death signals and unprotected mitochondria from blocked P53, triggered by loss of SIRT-1 control over apoptotic factors including P53 and FOXO3a. The data obtained from our work can reveal the magnitude of deviation resulting from using early-onset AD models rather than aging mice, hence serving as a reference to possible bias. The raised pathology about SIRT1 and uncontrolled apoptotic factors could serve as a potential target for AD study.

Keywords: ***Alzheimer's Disease, SIRT1, P53, FOXO3a***

1. Introduction

Alzheimer's Disease (AD) is a kind of incurable neurodegenerative disease with dementia as the most significant symptom and usually progresses 5-12 years until death. The causes of AD are complex and not fully understood, which involve genetics, pollution exposure, mental and physical trauma, diets, viral infections, and so on. Most cases of the disease are caused by a combination of these factors. Popular theories suggest the abnormal processes of Amyloid Precursor Protein and over-phosphorylation of microtubule tau proteins as major causes of AD [1,2].

Recently, studies about SIRT1 are gaining momentum for its close bond with AD. Located in the nucleus and cytoplasm, SIRT1 works as an NAD+-dependent deacetylase and plays a crucial role in manipulating anti-AD signals by ensuring neuron longevity, manipulating anti-inflammatory responses, repairing DNA damage, and suppressing pro-apoptotic factors like P53 and FOXO3a as shown in Figure 1[3,4]. The majority of the SIRT1 expressions in brain tissues are among neurons, namely, grey matter and cortexes, which is another reason why SIRT1 is so closely linked with Alzheimer's Disease in terms of the appearance of symptoms parallel to the loss of neurons [5]. Therefore, studies regarding AD pathology and potential treatments claim SIRT1 as a promising target protein for further investigation. Meanwhile, SIRT1 seems to provide new biomarkers for the disease. Research finds that there are increased expression levels but decreased SIRT1 activities due to the depletion of NAD+ [6]. Also, loss of SIRT1 would commonly lead to increased amyloidogenic processing of APP [7]. Some other studies suggest that loss of SIRT1 could be linked with working memory loss in the early progressions of AD, during which drops of SIRT1 levels and associated neuron deaths, and tau tangles are first detected among the two output centers of the hippocampus – CA1 and CA3 and adjoining areas [5]. Therefore, the field of cure developments discusses stimulators of SIRT1 levels as possible silver bullets to AD, which is not groundless -- many studies have shown that hyperactivity of SIRT1 leads to cognition improvement and delayed disease progression of AD [8]. In an AD mice model experiment, Significant cognitive improvements and reduction of AD biomarkers occurred after six weeks of resveratrol injection, the most effective activator of SIRT1[9].

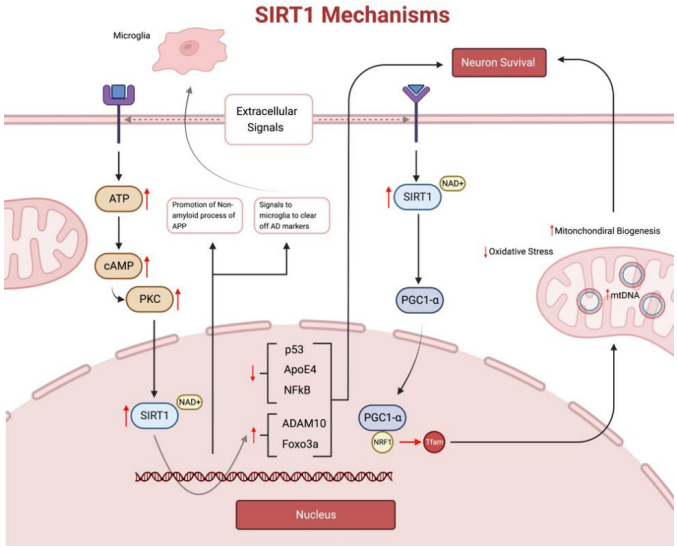

Figure 1. Summarized pathways of SIRT1 in promoting Anti AD signals.

A summary of SIRT1 functioning against/with other key molecular factors including P53, FOXO3ai, and so on in neurons. SIRT1 promotes neuron longevity, anti-inflammatory responses, and AD markers decomposition by up or down-regulation of downstream factors. Activating SIRT1 may be a potential approach to treat AD.

In experiments on AD pathology and treatments, mice models with APP expression since birth or early after birth are the most frequently used tools. By doing so, it's time-efficient to conduct the experiments since there is no need to wait till the mice grow old and then conduct the trials. However, compared to old mice, new-born ones have more vibrant metabolisms, more active DNA repairment systems, and higher levels of SIRT [10,11], which is essential for stable cell proliferation and body growth; while the inner conditions of old mice are much different, featuring slower metabolisms and weaker systems and more like the status of AD patients, who get the disease at old ages usually [12,13]. Therefore, the actual pathologic process is blurred with other variables such as differences in the expression of time-dependent genes and compensation effects as well [14,15]. As a result, it is not explained by evidence whether and how aging influences the progression of the disease, or whether the disease is based primarily on its distinctive pathology.

This study intends to investigate the complex molecular mechanisms related to SIRT1 and probe the differences in AD progressions caused by SIRT1 and aging. To do this, more accurate models with temporal regulation on SIRT1 and APP should be designed. Overall, four classes of models with SIRT1 and APP expressed at different times will be created.

Two hypotheses are raised in this report: first, the progression of AD would be significantly faster in the mouse model with APP triggered at 19 months old compared with the mouse with APP triggered 30 days after birth; second, sirt1 knockout will speed up the progression of the disease more than aging, while the disease progression will happen at a faster rate in sirt1 knockout mice models with APP expressed since birth (in terms of calculated indexes of the disease progression), compared to mice models with late APP expression but no sirt1 silence.

2. Materials and Methods

2.1 Preparation of SIRT1/Tet-Off System Vectors

Two plasmids are required for the implantation of SIRT1 with the Tet-Off system: one with SIRT1 and TRE binding site that we need to construct from a sample plasmid using SIRT1 CDS sequence as shown in Figure 2; the other a market-standardized tTa enzyme carrier in form of transfected bacteria stored in agar bars.

To prepare the SIRT1-carrying plasmid, mouse SIRT1 CDS is prepared by reverse transcription of mouse SIRT1 mRNA. Additional bases and BamHI restriction sites are added to each end of the derived sequence. The strand is then linked with the plasmid using the restriction enzyme BamHI and DNA Ligase I after amplification by PCR. After that, the SIRT1 vectors are transfected to a dish of E. coli using a water bath and a transfection kit. The tTa plasmid is directly derived after cultivation using a DNA extraction kit and centrifuge (13000rpm). The selection marker of both vectors is ampicillin.

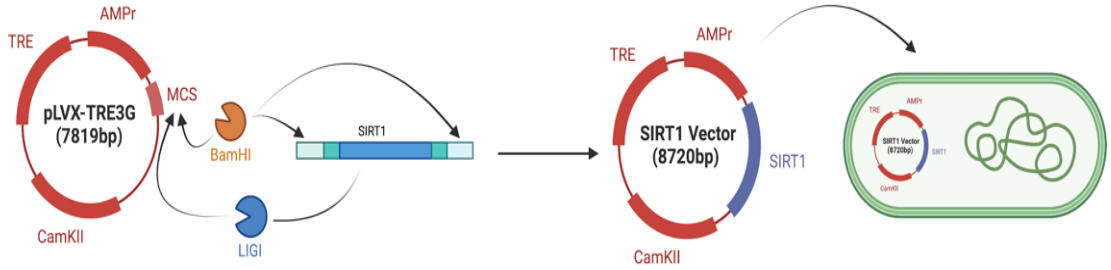

Figure 2. Gene Recombination of SIRT1 Plasmid.

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The design of the SIRT1 CDS vector. SIRT1 CDS including protective bases is implanted on a TRE plasmid driven by a CamKII promotor to manipulate SIRT1 expression in the brain. Primers F and R are designed to amplify such a structure.

Primer F: 5' cttttgtcttatacttggatccATGGCGGACGAGGTGGCG-3'

Primer R: 5'-ggcggccgcgggcccggatccTTATGATTTGTCTGATGGATAGTTTACAT-3'

2.2 Preparation of APP/CREer-LoxP Vectors

To allow spatially and temporally controlled APP expression in mice models, APP K595/M596L (Swedish mutation) gene is transfected into mice models with CREer-LoxP tamoxifen-dependent system as shown in Figure 3. The transfection involves two vectors: CREer-carrying plasmid (ERT2-CRE-ERT2) with promotor CamKII (to ensure spatial expression in grey matter and hippocampus) and Lox-Stop-Lox plasmid containing APP K595/M596. ERT-CRE-ERT2 is chosen since it is a CREer vector without a promotor and allows for control in temporal expressions. Lox-Stop-Lox plasmid would be used to ensure the silence of APP K595/M596 when no drug is delivered.

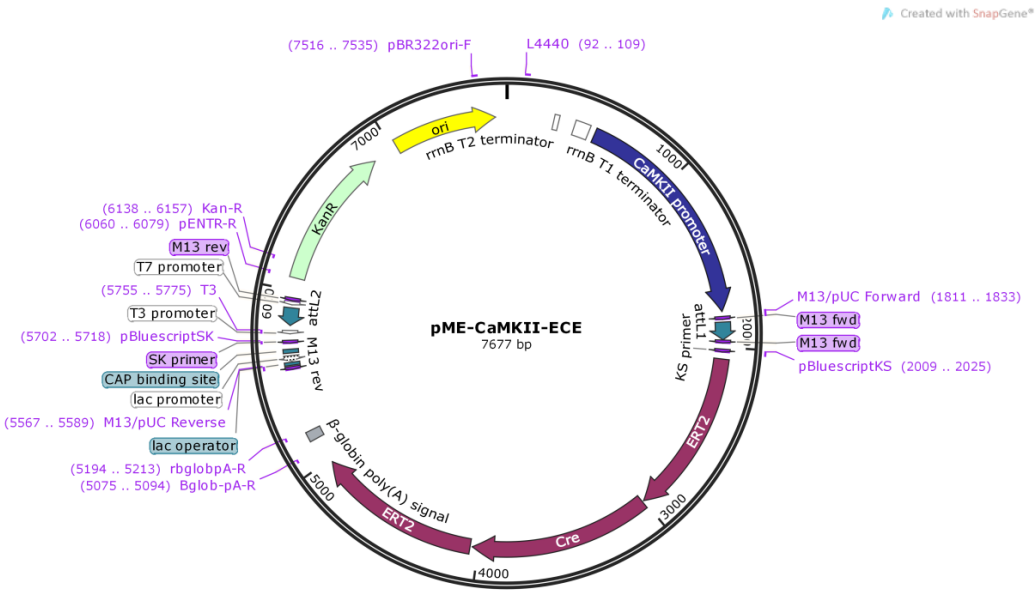

Figure 3. Gene Recombination of Aβ42 Plasmid.

The design of the Aβ42 vector. Aβ42 mutation CDS including protective bases is implanted on a CRE plasmid driven by a CamKII promotor to manipulate Aβ42 expression in the brain.

2.3 Preparation of Transgenic Mouse

CamKII/CREer vector and LoxP/APP vectors are microinjected into two sirt1 knockouts (to eliminate SIRT1 background expression) zygotes separately. The cells are then implanted back into two parents with sirt1 KO. Offspring from the parents are bred at maturity and two second-generation zygote cells are extracted. The SIRT1/TRE and the vectors are transfected into these zygote cells separately and implanted back into another two sirt1-KO parents. The offspring are crossed again to give birth to SIRT1/APP double transgenic mice. In the

double transgenic mice, both target genes' activities are drug-dependent: SIRT1 is present but can be silenced through Tetracycline administration; APP is also present and can be irreversibly activated by a single dose of Tamoxifen. The four transgenic models with different SIRT1/APP temporal expressions would be derived from those double transgenic mice with SIRT1 transfection in the Tet-Off system, and APP in CREer/LoxP switch. Activation of both systems is drug-dependent, with doxycycline for Tet-Off (SIRT1 associated), and Tamoxifen for CREer/LoxP (APP associated).

2.4 Temporal Regulations of Gene Expressions in Four Different Models

Five conditions of different temporal gene expressions of SIRT1 and APP would be generated through the administration of drugs at different times (Tetracycline for SIRT1 and Tamoxifen for APP). The conditions are SIRT1 normal expression (No Tetracycline administration throughout life), SIRT1 late silence (Tetracycline administration 19 months after birth), SIRT1 early silence (Tetracycline administration 30 days after birth and throughout life), APP early expression (Tamoxifen activation 30 days after birth), APP late expression (Tamoxifen activation 19 months after birth). Combining the conditions, four transgenic models are produced: A, B, C, and D. Both model A and B has normal SIRT1 expressions, but late APP expression in model A, and early APP expression for model B. In model C, SIRT1 is silenced late, APP is turned on at the same time SIRT1 is silenced. In the last model, D, SIRT1 is silenced early after birth as APP is turned on simultaneously.

2.5 Paired Models

To analyze the effects of aging on AD, we put model A against model B. SIRT1 is normally expressed in both models, but model A has APP expressed 19 months after birth while model B has APP expressed 1 month after birth. To analyze the effects of SIRT1 on AD, we compare two pairs of models: model B versus model D, and model A versus model C. In the cases of models B and D, both mice have APP expressed since birth, but one with SIRT1 expression silenced since birth, the other SIRT1 expressed normally. In the case of A and C, both mice will not have APP expressed till 19 months. However, mouse C has SIRT1 silenced at the same time as APP is expressed, and Model mouse A has SIRT1 normally expressed. Such pairings of models are shown in Figure 4.

Finally, with data derived from the previous experiments, the effects of aging and loss of SIRT1 on AD progression could be compared by putting model A against model D. The results derived from the four groups would be compared and checked for our hypothesis. The results will be measured in terms of over-phosphorated tau, amyloid plaques accumulations, proapoptotic factor levels like FOXO3a and P53 using western blot, also behavioral tests like Morris-water maze and the eight-armed maze test. Activities of SIRT1, FOXO3a/P53 would be observed in vitro through brain tissues with immunostaining in all regions of the tissue. We assume that P53 and FOXO3a levels increase as DNA errors accumulate through time, with their levels affected by many more factors. There will be eight mice for each model and seven slices of brain tissue collected (two sagittal and two coronals, collected before APP activation) from the same model source, which would share parallel drug manipulations with the models.

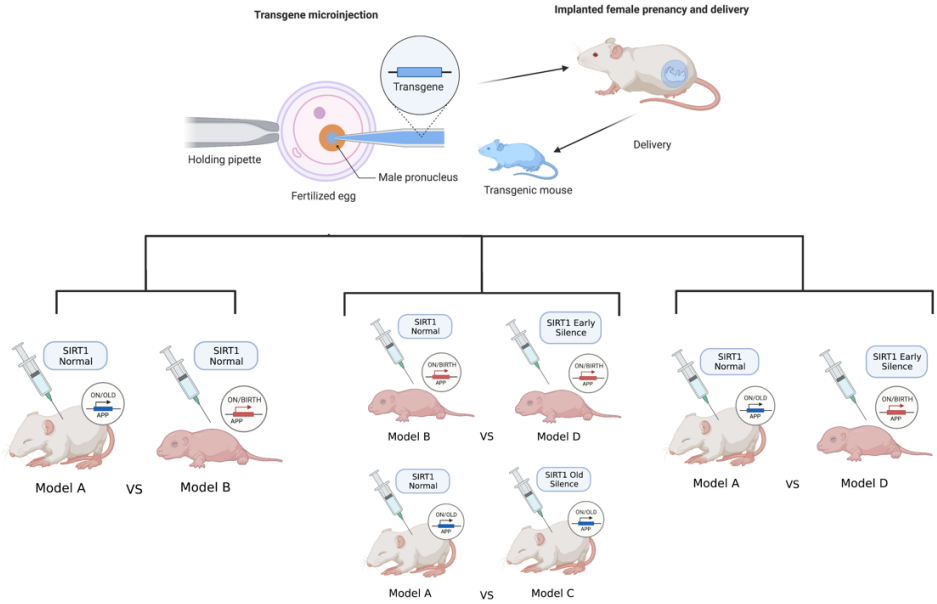

Figure 4. Paired Mice Models.

Four models are constructed by providing the mice with Tamoxifen and Tetracycline at different stages in their life. The models are arranged into four pairs to compare the effect of aging and SIRT1 knockdown/knockout on AD progression.

2.6 Protein Tracking and Behaviour Experiments

Fluorescent proteins have been widely used as biomarkers in cell studies in the past decades. The diversity of the GFP (Green Fluorescent proteins) family allows us to detect multiple proteins in this experiment. By attaching different types of fluorescent proteins to targeted AD markers through immunostaining, we could reveal the images of interactions between Aβ 42, p-tau, FOXO3a, and p53 under super-resolution Microscopy as shown in Figure 3. The specific pairing of AD markers is shown in Table 1. AMPK and DAF-16 [31] [32] are two aging biomarkers being tracked that play significant role in homeostasis of cellular energy and the prevention of senescence. AMPK and DAF should be inversely proportional to age.

Table 1. AD Markers in Track and Matched Fluorescent Marker.

AD Marker	Fluorescent Marker (Color/EX/EM)
Aβ 42	DsRed2 (Red, 563nm, 582nm)
p-tau	EYFP (Yellow, 513nm, 527nm)
FOXO3a	mTagBFP2 (Blue, 400nm, 500nm)
P53	EGFP (Green, 488nm, 507nm)

Table 2. Aging Markers in Track and Matched Fluorescent marker

Aging Marker	Fluorescent Marker (Color/EX/EM)
AMPK	ECFP (Cyan, 439nm, 475nm)
DAF-16	mOrange (Orange, 548nm, 562nm)

Note: Each of the markers is matched by a fluorescent dye that is shown at a specific wavelength of light exposure.

Multiple brain slices including sagittal and coronal sections above the hippocampus and other coordinates would be collected from each group. Furthermore, the concentration, rate of accumulation, activity levels, and spatial distributions of the four AD markers would be measured using western blot and ELISA.

Behavioral tests like Morris-water Maze, Eight-armed Maze Test, Rotarod Test, and other behavioral or recognition tasks are included. The data collected from those experiments would be compared with activities and levels of SIRT1, FOXO3a or P53 observed through corresponding brain slices in the groups.

2.7 Proposed Statistical Analysis

Correlations between variables in every experimental group wound be examined with Spearman rank correlation test [33]. There are four main variables measured: SIRT1 level, degree of aging, degree of AD progression and cognitive performance. First, SIRT1 level is an independent variable measured in terms of activity and concentration of SIRT1 revealed by Western Blot. Then, aging is another independent variable measured in terms of levels of biomarkers listed in Table 2 in reference to age of the model. The first dependent variable, AD progression, is measured in terms of the number of accumulations of Table 1 markers. The other dependent variable, cognitive performance, would be observed through various trials of behavioral experiments including Morris's water maze and eight-armed maze test. To differentiate effects of aging from SIRT1 deficiency on progression of the disease, all data collected from the models would undergo paired student's t-test [34]. In the tests, $p<0.05$ would be considered as statistically significant.

3. Possible Results

3.1 *Aging and AD Progression*

Based on research and experiments on the Anti-AD and aging mechanisms of SIRT1, advanced mouse age was shown to impact the density of neurotic tau inclusions and caused significant increases in the ventral hippocampus and corpus callosum in the models observed [16]. So, it's quite predictable that the models with APP turned on at old age would have faster rates of AD progression, which means faster and more intense accumulation of amyloid and tau, poorer cognitive and memory performance, and higher accumulation of pro-apoptotic signals, provided that SIRT1 levels are normally expressed in A and B. That is to say, the progression of AD in mouse A will be faster than in mouse B.

3.2 *Loss of SIRT1 and AD Progression*

When we compare model B with model D and compare model A against model C, the results are also predictable according to existing relevant experiments. When SIRT1 is normally expressed, AD progresses more slowly. When SIRT1 is silenced, AD progresses more quickly. That means, model D has faster progression compared with B; and model C has faster progression compared with model A.

The underlying mechanisms have been proposed by some of the research in the field. For example, SIRT1 overexpression in levels reduces Aβ production and Aβ plaques, while SIRT1 deletion increased Aβ levels [17]. Such a phenomenon is caused by SIRT1 deacetylation of Retinoic Acid Receptor beta (RARβ) and activation of α-secretase ADAM metallopeptidase domain

10 (ADAM10); consequently, α-secretase levels increase in neurons, so α-secretase performed APP restrictions occur at a higher rate. SIRT1 reduction is also found parallel to tangle accumulation, in terms of both levels and activities in the parietal cortex of AD patients. Further research in the same study confirmed that SIRT1 in the cortical cortex was decreased in AD patients only but not in individuals with mild cognitive impairment [5].

3.3 *Comparing the Effect of Aging & Loss of SIRT1 on AD Progression*

For the last pair, we put model A against model D. This scenario is the most complex and intriguing one. Regarding the rate of AD progression, we have deduced that model A is faster than model B, while model D is also faster than model B. In terms of AD resistance, A has the advantage of normal SIRT1 mixed with the disadvantage of aging at which time APP is expressed. While mouse D has the disadvantage of SIRT1 absence mixed with the advantage of youth at which time APP is expressed. So, the actual result that which model would have a faster rate of AD progression is up to whether the lack of SIRT1 or aging will play a more dominant role in AD progression.

Although the results are yet to know, we can classify the possible outcomes into three categories and discuss the underlying information behind each of them:

Rates of progression are about the same in Model A and Model D

The rate of progression in model A is Slightly/ Significantly slower than in Model D

The rate of progression in model A is Slightly/ Significantly faster than in model D

The first category of results represents that aging and lack of SIRT1 are contributing an approximately equal share of influence on AD progression. In this case, rates of pathological markers (plaques and tangles) formation observed in the groups would be about the same. We expect higher levels of expression of P53 and FOXO3a but lower levels of average activities in model A, and lower levels of expression of P53 and FOXO3a but higher average activities in model D. Overall, the two groups of models have P53 and FOXO3a functioning in approximately the same degree. The cognitive scores would be about the same in the two groups.

The second category of results represents that lack of SIRT1 contributes more to AD progression than aging. If the result falls into this category, rates of pathological markers (plaques and tangles) formation observed in group model D would be faster than in group model A. In group model A, P53 and FOXO3a levels are expected to be higher, while their activities should be moderately lower. P53 and FOXO3a levels are expected to be lower in group model D, while their activities are expected to be much higher. The effects of level and activity combined, Model Ds have higher overall FOXO3a/P53 functioning levels. The average cognitive scores of group model Ds should be lower than group model A's.

The third category of results represents that aging exerts a greater effect on AD progression compared to the loss of SIRT1. The results obtained show that rates of pathological markers (plaques and tangles) formation observed in group model A are greater than in group model D. P53 and FOXO3a levels would be much higher in group model A, while their average activities would be slightly lower or about the same as the other group. P53 and FOXO3a levels would be lower in model Ds, while their average activity could be much higher. The factors of expression levels and activities combined, Model A has higher levels of P53/FOXO3a functioning than model D. The cognitive scores would be lower in model A.

Decreasing levels of AMPK and DAF-16 are recognized as signs of aging in all models. The levels of the two markers are expected to be lower in models A and C, which are 19th old when Aβ expression is turned on. Models B and D, which has Aβ turned on at 1st month after birth, are expected to have higher level of the two markers. Although SIRT1supression have some effects on the gradient of AMPK, DAF-16 level fall against time, we believe that such a factor would not disrupt the clear relationship between aging, AMPK and DAF-16 since age plays a more dominant role in the capability of the cell metabolism intensities than SIRT1 [35] [36] [37]. Therefore, AMPK and DAF-16 level changes in reference to time could be directly used as estimates of aging degrees and be compared against SIRT1 levels to differentiate the effects of aging on AD progression from SIRT1.

4. Discussion

If the results support that loss of SIRT1 exerts equal or more significant effects in AD promotion than aging, it could only happen under the condition that SIRT1 has its specific mechanism on AD progression which is independent of aging (distinctly related to AD in molecular mechanisms). Therefore, when SIRT1 is silenced, a lot of the AD-triggering mechanisms comes hyperactive. One of the mechanisms is the imbalance between apoptosis-inhibiting systems and apoptosis-promoting systems including P53. P53 is a key promoter of cell apoptosis in humans. It detects DNA mutations and tries to repair them if possible. While if the mutations cannot be repaired, P53 activates cell apoptosis, which is supposed to be an advantage of body cells in that the amplification of

mutations through cell replications would be prevented. However, a high level of cell apoptosis could bring a massive amount of neuron loss, which is unrecoverable and unnecessary as well since neurons could not replicate and several neurons could still function with a certain number of damages. Furthermore, neuron death could bring synapse degeneration, and apoptosis of a single neuron is likely to lead to apoptosis of other neurons through synapse degeneration and neuron excitotoxicity [18]. Therefore, a high level of P53 in neurons could cause irreversible and unnecessary neuron deaths. SIRT1 is a posttranscriptional suppressor of P53 activity and can suppress apoptotic processes by inhibiting P53. Similar relationships exist between SIRT1 and other pathways like FOXO3a and so on [19]. When SIRT1 activities go down, control over apoptosis signals weakens, and neuron death signals increase, hence AD progresses fast. Loss of SIRT1 could also make cells more vulnerable to oxidative stress hence pushing AD progression -- these mechanisms are connected to apoptosis signals discussed before. ROS activates cytoplasm SIRT1 protein, which binds to P53 and deacetylates it [19,20], leading to blocked P53 nuclear translocation and hence inhibited apoptosis. In such a scenario, deacetylated P53 transfers into mitochondria and establishes further protection against AD: mitochondrial stress prevention, also neural protection, especially in dopaminergic neurons. Such a mechanism might be the cross-link between Alzheimer's disease and Parkinson's disease, which has the loss of dopaminergic neurons as the main cause. When the SIRT1 level goes down, mitochondrial, and dopaminergic neuronal protections weaken, and apoptosis is persuaded. All these mechanisms work as potential pathologies related to SIRT1 loss-promoted AD progression. Such mechanisms have not been fully illustrated and consist of inviting future directions, around which potential drugs could be researched: SIRT1 activators, isozymes, artificial stimulators targeting neurons to perform cell cycle arrest, and P53 or FOXO3a suppression.

Another possible reason to suspect that loss of SIRT1 contributes to AD progression is that decrease in SIRT1 hippocampal level could accelerate AD progression and promote working memory loss [21]. The mechanisms behind this could partially result from apoptosis, decreased neuron plasticity, and Hippocampal nerve regeneration due to loss of SIRT1, as discussed before [23,24]; when SIRT1 is lost significantly, over-phosphorated tau tangles accumulation in local regions around CA1 and CA3 is expected to be observed, which supports previous discussions [5]. If we derive decreased memory performance through the behavioral test trials in our study, the idea that loss of SIRT1 leads to working memory loss can be further testified, which can highlight SIRT1 as a target to develop drugs that detain early AD progressions and improve working memory of patients.

If aging is proven to have significant effects on accelerating AD progression compared to the loss of SIRT1, the progression of AD would likely be more of a time-reliant process including the accumulations of pathway errors, the weakening of repairment systems, expression of genes through time and the buildup of DNA damages [25-28]. Therefore, on the one hand, more focus should be put on comprehensive anti-aging measures to find solutions to AD; On the other hand, the result suggests that the effects of aging in AD mice model studies are significant and that mice models with early expression of AD pathological markers could bring unignorable distortions [29,30]. In that case, mice models with AD progression through natural aging should be utilized more widely in research henceforth.

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