SPANDIDOS PUBLICATIONS

Analysis of differentially expressed genes in early- and late-stage APPsw-transgenic and normal mice using cDNA microarray

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Abstract. The complexity of Alzheimer's disease (AD) has made it difficult to examine its underlying mechanism. A gene microarray offers a solution to the complexity through a parallel analysis of most of the genes expressed in the brains from AD-transgenic mice. In our previous study, a total of 52 differentially expressed genes were identified in 18-month-old *APPsw*-transgenic mice compared to age-matched normal mice. We extended our work to better understand the relevant gene profiles from both early- and late-stage transgenic and normal mice. To accomplish this, cDNA microarray was used with the large-scale screening of the brain mRNA from transgenic and normal mice of 1 and 18 months of age. We identified a total of 48 genes, 6 up-regulated and 42 down-regulated, differentially expressed with a significant degree of induction and reduction in the brains from moderate 18-month-old transgenic mice compared to 1-month-old transgenic mice. In parallel, a total of 40 differentially expressed genes, 6 up-regulated and 34 down-regulated, were also found in the brains from moderate 18-month-old normal mice compared to 1-month-old normal mice. Thus, differentially expressed genes upon APPsw overexpression and the aging process are useful targets through which investigators can choose genes of particular interest. In the future, it will be necessary to study the function of differentially expressed genes, which are targets for developing drugs, using pharmacoproteomics.

Introduction

The massive accumulation of abnormal fibrous amyloid ß-protein (Aß) in the brain is the most common cause of Alzheimer's disease (AD), which is accompanied by memory and cognitive impairment. Aß is deposited as extracellular senile plaques, composed of the 39 to 43 amino acid-long peptides derived from the amyloid precursor protein (APP) by cleavage with ß- and γ-secretase. An α-secretase cleaves the middle of the Aß region releasing a secreted ectodomain containing the first 16 amino acids of the Aß (sAPPα). A ß-secretase [ß-site APP cleaving enzyme (BACE)] cleaves between Met 671 and Asp672, producing a membrane-bound COOH-terminal fragment of the ßAPP (sAPPß), which is the substrate of γ-secretase (1). The sAPPß can be transported back to the cell surface where the final γ-secretase cleaves the end of the sAPPß, resulting in the Aß release with a secreted ectodomain (2). This Aß is a key event in the pathogenesis of AD. Thus, AD is a complex, progressive disorder resulting in increased cognitive impairment.

With the understanding that AD is hampered by its complexity, there currently exists new genomic technology, such as complementary DNA (cDNA) microarray. cDNA microarrray is one potential technology that can be used to explore the interactions between gene expression and disease. This approach is characterized by high-density arrays of cDNA sequences, bound to a structural support such as glass. The genome-wide expression profiling of thousands of genes provides rich data on genes that best characterize the diseased state. One of the first microarrays showed about 3 times as many known down-regulated genes in comparison to amplified RNA from normal and tangle-bearing neurons of AD (3). There were also several changes in genes under neurotransmitter regulation observed in the superior temporal gyrus from cDNA microarray analysis (4). Furthermore, the down-regulation of several gene transcript families, including genes for cytoskeletal mobility and protein and fatty acid metabolisms were observed using cDNA microarray (4). In the Alzheimer's disease model of *C. elegans*, 67 up-regulated and 240 down-regulated genes were identified using cDNA microarray (5). It has also been reported that the amyloid-containing region of the transgenic brain, co-expressing amyloid precursor protein (*APP*) and *presenilin-1*, had high levels of inflammation-associated gene expression, although the most intriguing region of the gene expression profile was selectively decreased (6).

We previously produced transgenic mice expressing neuron-specific enolase (*NSE*) promoter-controlled *APPsw*

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(7). It was demonstrated that cognitive deficits along with Aß-42 depositions were shown at 12 months of age in transgenic mice. These mice provide an important resource in gaining insight into the potentially overexpressed effect of *APPsw* on the modulation of genes for AD, which is central to understanding the complexity of AD. Moreover, a total of 52 differentially expressed genes, which are associative AD-related phenotypes, were found in *NSE/APPsw*-transgenic mouse brains (18 months old) compared to non-transgenic brains of identical age using cDNA microarray (8). However, the differences in gene profiles from early- and late-stage transgenic and normal mice have not been characterized

In this study, the previous study was extended in order to elucidate the relevant gene profiles using cDNA microarray technique in a large-scale screening of brain mRNA from 1- and 18-month-old *APPsw*-transgenic and normal mice. Here, 18-month-old transgenic mice were chosen as they may have the Aß-42 plaques and neuronal loss corresponding to AD pathology, and in addition, there may be neuronal loss in normal aging mice. This study identified 48 and 40 differentially expressed genes with a significant degree of induction and reduction in brains exhibiting moderate AD and aged brains (18 months of age) compared to those in early-stage transgenic and normal mice (1 month of age).

Materials and methods

Mice. Transgenic lines, expressing the *NSE/APPsw* fusion gene, were established by backcrossing the founder mice with the parental strain of C57BL/6 mice (7). Transgenic and normal mice were handled in accredited Korea FDA animal facilities in accordance with AAALAC International Animal Care policy (Accredited Unit-Korea Food and Drug Administration, unit no. 000996). Mice were housed in cages under a strict light cycle (lights on at 06:00 and off at 18:00). In addition, all mice were given a standard irradiated chow diet (Purina Mills, Inc) *ad libitum*, and maintained in a specific pathogen-free state.

RNA isolation. The brains were used for isolating total RNA using Trizol™ reagent (Invitrogen, Carlsbad, CA) and purified using an RNeasy Total RNA Isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA quantity and quality were checked by a spectrophotometer, and the integrity was assessed on a denaturing 0.8% agarose gel. Since the brain environment is in an acidic condition (<pH 6.5) with a prolonged agonal phase, the purified RNA message was checked on an agarose gel to assess degradation prior to the synthesis of cDNA (9). There was no degradation of the RNA message profile from the brains.

cDNA microarray. The profiling of gene expression was analyzed with a Tween-Chip Mouse-7.4K (Digital Genomics, Seoul) consisting of 7616 mouse cDNA clones. For the synthesis of proof cDNA, RNA was used to generate Cy3- and Cy5-labeled cDNA, and probe cDNA microarray slides as described (10). The GeneScript II (Gibco BRL, Rockville,

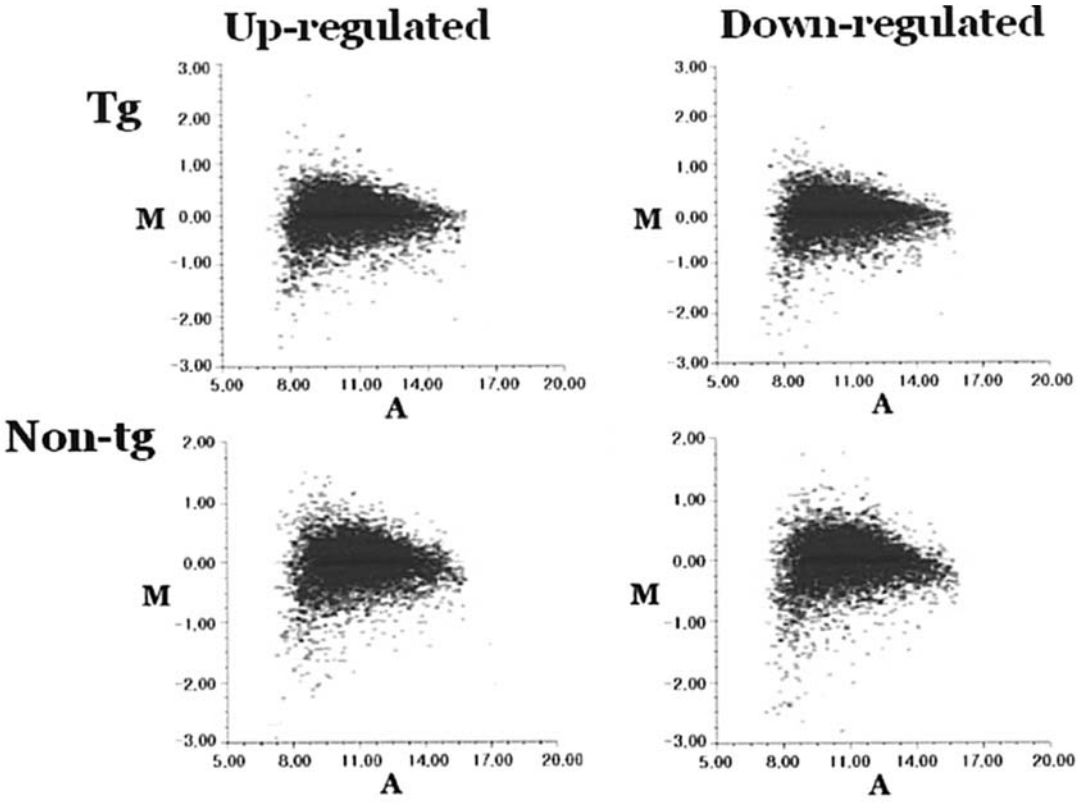

Figure 1. MA plots of cDNA microarray hybridization. Gene expression signal of mouse brains. MA plots were used to represent R, G data, where M=log2R/G, and A=log2(RxG). M, expression ratio; A, signal intensity; G, stronger 1-month-old mouse sample hybridization (Cy3); R, stronger 18-month-old mouse sample hybridization (Cy5). One- and 18-month-old transgenic (Tg) and non-transgenic mice (Non-tg) are indicated (up-regulated genes and down-regulated genes).

iene profiles in transgenic mice.

A. Down-regulated genes.

Gene symbol	Description	GenBank accession no.	Fold change
Cell growth and maintenance			
Abca2	ATP-binding cassette, sub-family A (ABC1), member 2	AI413825	-2.14
Chrna4	cholinergic receptor, nicotinic, α polypeptide 4	AI854369	-3.17
Col1a1	procollagen, type I, α 1	AI425767	-2.57
Col1a2	procollagen, type I, α 2	AI841886	-2.54
Col3a1	procollagen, type III, α 1	AI842703	-2.67
Gsn	gelsolin	AI850094	-3.09
Hao3	hydroxyacid oxidase (glycolate oxidase) 3	AA116296	-2.73
Krt1-10	keratin complex 1, acidic, gene 10	AI325191	-2.11
Mg29	mitsugumin 29	AI427574	-2.91
Mpz	myelin protein zero	AA080181	-3.38
Myo7a	myosin VIIa	AI874956	-3.16
Nup160	nucleoporin 160	AI426942	-4.42
Slc23a1	solute carrier family 23 (nucleobase transporters), member 1	AI428174	-2.48
Cell death			
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	AI426523	-2.80
Apoptosis			
Dock1	dedicator of cyto-kinesis 1	AI267063	-2.02
Cell cycle			
Tacc3	transforming, acidic coiled-coil containing protein 3	AI429136	-2.34
Transcription			
En2	engrailed 2	AI844870	-2.18
Gata3	GATA binding protein 3	AI428208	-4.21
Harp	harmonin interacting ankyrin repeat containing protein	AI452087	-5.20
Ncoa6	nuclear receptor coactivator 6	AA517662	-2.47
Ppargc1a	peroxisome proliferative activated receptor, γ, coactivator 1 α	AF049330	-2.31
Stat5a	signal transducer and activator of transcription 5A	AA763337	-2.14
Immune response			
Adn	adipsin	M11768	-3.33
Chi3l3	chitinase 3-like 3	AI505981	-2.23
Daf1	decay accelerating factor 1	AI120685	-3.82
Response to stress			
Eif2ak3	eukaryotic translation initiation factor 2 α kinase 3	AI427929	-3.23
Ucp3	uncoupling protein 3, mitochondrial	AF053352	-3.14
Signal transduction			
Adrb2	adrenergic receptor, ß 2	BC032883	-2.35
Adrb3	adrenergic receptor, ß 3	AF193027	-2.08
Car8	carbonic anhydrase 8	AI838156	-2.77
Gabra6	γ-aminobutyric acid (GABA-A) receptor, subunit α 6	AI839865	-2.57
Gna13	guanine nucleotide binding protein, α 13	AI426467	-3.40
Il10ra	interleukin 10 receptor, α	AI173487	-4.59
Map3k6	mitogen-activated protein kinase kinase kinase 6	AI155236	-2.04
Pcp2	Purkinje cell protein 2 (L7)	AI843793	-2.77
Ppp1r1a	protein phosphatase 1, regulatory (inhibitor) subunit 1A	AI325468	-2.94
Psen2	presenilin 2	AI851854	-2.71
Rai3	retinoic acid induced 3	AA096667	-3.50
Rgs12	regulator of G-protein signaling 12	AI450971	-3.94
Spnb2	spectrin ß 2	AI448706	-2.12
Tnf	tumor necrosis factor	M13049	-2.13
Tyk2	tyrosine kinase 2	AI452085	-2.61

Table I. Continued.

B. Up-regulated genes.

Gene symbol	Description	GenBank accession no.	Fold change
Cell growth and maintenance			
Gstk1	glutathione S-transferase κ 1	AI323948	2.10
Stxbp3	syntaxin binding protein 3	AI528529	2.25
Apoptosis			
Pglyrp1	peptidoglycan recognition protein 1	AI507116	2.41
Trp53inp1	transformation related protein 53 inducible nuclear protein 1	AI835817	2.39
Cell cycle			
Nbn	Nibrin	AI850789	2.39
Immune response			
Orm1	Orosomucoid 1	AI117779	2.65

MD) in combination with an oligo dT18 primer (Ambion, Austin, TX) were used to synthesize labeled cDNA from total RNA. After removing the unincorporated fluorescent nucleotide, the Cy3- and Cy-5-labeled cDNA proofs were mixed together and hybridized overnight at 65˚C on microarray slides. After hybridization, the solution was removed, and the slides were washed twice with 2X SSC containing 0.1% SDS for 5 min at 42˚C, once with 0.1% SSC containing 0.1% SDS for 1 min at room temperature, and finally with 0.1% SSC for 1 min at room temperature. The slides were then dried by centrifugation at 650 rpm for 5 min. Hybridized arrays were scanned with a Scanarray lite (Packard Bioscience, Boston, MA) and analyzed by GenePix Pro 3.0 software (Axon Instruments, Union City, CA) to obtain a gene expression ratio (Cy-3 vs Cy-5). A typical false color image is depicted, showing red signals for stronger diseased sample hybridization, green signals for stronger control sample hybridization, and yellow signals for relatively equal transcript abundance.

Analysis of cDNA microarray data. Data analysis was performed using the GenePlex (Istech, Inc., Korea). Logged gene expression ratios from the fluorescent intensity of each spot were normalized by LOWESS (locally weighted scatter plot smoother) regression. Data and statistical significance of the differential expression were performed by computing a q-value for each gene (11).

Results

MA plots. To compare differential expression, microarray data was normalized with the lowest of the experiments repeated in triplicate. The normalized log ratios of varied genes in each experiment were used to analyze the relativity between two independent experiments. Relative changes were then calculated and were plotted on the basis of mean value intensities of each spot in three experiments (Fig. 1).

Gene expression profiles in APPsw-transgenic mice. Gene expression profiles of the brains from 18-month-old *APPsw*-transgenic mice were compared to those of 1-month-old *APPsw*-transgenic mice using cDNA microarray. Of the 7616 genes, 6 genes in the 18-month-old *APPsw*-transgenic mouse brains were significantly up-regulated (Table IB) and 42 genes were down-regulated compared to those in the 1-month-old *APPsw*-transgenic mice (Table IA). The 6 genes with increased expression and the 42 genes with decreased expression include the proteins and enzymes related to cell growth, cell cycle, cell death, immune system, signal transduction, transcription, and stress.

Gene expression profiles in normal mice. Gene expression profiles of the brains from 18-month-old BDF1 mice were compared to those of 1-month-old BDF1 mice using cDNA microarray. Of the 7616 genes, 6 genes in the 18-month-old mouse brains were significantly up-regulated and 34 genes were down-regulated compared to those in the 1-month-old mice (Table IIA and B). The 40 genes include the proteins and enzymes related to cell growth, cell cycle, cell death, immune system, signal transduction, transcription, and stress.

Discussion

In this study, late-stage transgenic mice overexpressing APPsw and normal mice were used to address a hypothesis that genes for AD and aging modulate in a large scale compared to early-stage transgenic and normal mice. The approach of cDNA microarray has allowed us to offer critical insight that may have an impact on the complexity of AD and the aging process.

We identified that 6 genes from the brains of 18-month-old transgenic mice were significantly up-regulated and 42 genes were down-regulated compared to those in the brains of 1-month-old transgenic mice of the 7617 mouse cDNA clones (Twin Chip Mouse-7.4K). A total of 48 genes are involved in the areas of 7 categories relating to growth, cell cycle, apoptosis, signal transduction, transcription, immune system, and stress. These changes in gene expression resulted from the overexpression of *NSE*-controlled *APPsw*

Gene profile in non-transgenic normal mice.

A. Down-regulated genes.

Gene Symbol	Description	GenBank accession no.	Fold change
Apoptosis			
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	AI426523	-2.37
Cd5l	CD5 antigen-like	AI047839	-2.32
Egln3	EGL nine homolog 3 (*C. elegans*)	AA717115	-2.05
Cell cycle			
Cdc25a	cell division cycle 25 homolog A (*S. cerevisiae*)	AI848278	-2.32
Cell growth and maintenance			
Mg29	mitsugumin 29	AI427574	-3.93
Sec15	SEC15 homolog (S. cerevisiae)	AI429813	-3.72
Col1a2	procollagen, type I, α 2	AI841886	-3.39
Col1a1	procollagen, type I,α 1	AI425767	-2.91
Hbb-bh1	hemoglobin Z, ß-like embryonic chain	AI385765	-2.91
Lrp10	low-density lipoprotein receptor-related protein 10	AI448458	-2.89
Adamts2	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 2	W71229	-2.67
Mpz	myelin protein zero	AA080181	-2.66
Yes	Yamaguchi sarcoma viral (v-yes) oncogene homolog	AI323763	-2.39
Col3a1	procollagen, type III, α 1	AI842703	-2.38
Ly75	lymphocyte antigen 75	AA867690	-2.28
Csng	casein γ	AI121961	-2.17
Gsn	gelsolin	AI850094	-2.15
Trappc3	trafficking protein particle complex 3	AI452141	-2.11
Slc23a1	solute carrier family 23 (nucleobase transporters), member 1	AI428174	-2.06
Immune response			
Rag1	recombination activating gene 1	AA153443	-3.38
Response to stress			
Eif2ak3	eukaryotic translation initiation factor 2 α kinase 3	AI427929	-3.53
Orm1	orosomucoid 1	AI117779	-2.92
Tacc3	transforming, acidic coiled-coil containing protein 3	AI429136	-2.02
Signal transduction			
Catnal1	catenin α-like 1	AW319897	-5.08
Pcp2	Purkinje cell protein 2 (L7)	AI843793	-4.53
Tyk2	tyrosine kinase 2	AI452085	-3.97
Gna13	guanine nucleotide binding protein, α 13	AI426467	-3.34
Mc2r	melanocortin 2 receptor	AI153955	-2.86
Car8	carbonic anhydrase 8	AI838156	-2.85
Hrh3	histamine receptor H 3	AI452091	-2.57
Adrb2	adrenergic receptor, ß 2	AA111732	-2.37
Transcription			
Harp	harmonin interacting ankyrin repeat containing protein	AI452087	-3.86
Gata3	GATA binding protein 3	AI428208	-2.89
Asxl1	additional sex combs like 1 (*Drosophila*)	AI837616	-2.14

in 1- and 18-month-old transgenic mice. The genes associated with AD are in the area of 7 categories. First, genetic mutations in genes lead to the risk of AD. The risk of AD was increased by a mutation in the brain-specific *Abca2* gene, which is known to participate in cholesterol and phospholipid transport (12). Also, a mutation in the *Chrna4* gene coding for the most widely distributed nicotinic receptor subtype II is associated with the late-onset of AD

Table II. Continued.

B. Up-regulated genes.

Gene Symbol	Description	GenBank accession no.	Fold change
Cell cycle			
Cetn2	centrin 2	AI326150	2.01
Gmnn	geminin	AI504205	2.82
Cell growth and maintenance			
Slc16a1	solute carrier family 16 (monocarboxylic acid transporters), member 1	AI427128	5.46
Signal transduction			
Tlr4	toll-like receptor 4	AA175249	2.15
Gngt2	guanine nucleotide binding protein (G protein), γ transducing activity polypeptide 2	AI154009	4.39
Transcription			
Cops2	COP9 (constitutive photomorphogenic) homolog, subunit 2 (*Arabidopsis thaliana*)	AI842912	2.03

(5). In *gelsolin* the mutation corresponds to codon 187, and the mutated gene is expressed in the amyloid fibril at residue 15 (13). Moreover, a deletion in the *GSTT1* gene enhanced susceptibility to AD (14). A polymorphism in the tumor necrosis factor (*TCF*), in association with apolipoprotein E (*APOE*) was reported to increase the AD risk (15). Secondly, changes in the concentration or activity of genes have been shown to result in AD. Loss, late-developed, granular degeneration, and breakdown in myelin lead to the pathogenesis of AD (16-18,24). It was demonstrated that reduced sensitivity/density or activation in adrenergic receptor 2 (*Adrb2*) contributes to the stress in caregivers of AD and the neuronal apoptosis in AD (20,21). In carbonic anhydrase, dysfunction impairs cognition and is associated with AD (22). The protein level of γ-aminobutylic acid (GABA) receptor subunits-1 or -5 in the hippocampus was increased in displaying the severity of AD neuropathology (19,21). Modulation of the mitogen-activated protein kinase (MAPK) kinase kinase, a member of the MAPK superfamily, may be crucial in the neuropathology of AD, since MAPK activation is a regulator of the formation of the plaques leading to AD (23). There are three enzymes in retinoic acid-induced 3 (Rai3), i.e. phospholipase A2, phospholipase C, and phospholipase D. Of the Rai3 enzymes, downstream transcriptional regulation of phospholipase A2-mediated signal transduction is involved in AD (24). When tissues from AD brains were immunostained with anti-spectrin ß2, ß-amyloid plaques were significantly stained in the cortical parenchyma (25). A decrease in glutathione S-transferase (GST) activity was observed in all areas of AD (26). In addition, syntaxin 1A was identified as a novel binding protein for presenilin 1 (27). Other genes listed may also be associated with AD pathogenesis, but it is unknown.

Six genes from the brains of 18-month-old mice were significantly up-regulated and 34 genes were down-regulated compared to those in the brains of 1-month-old mice of the 7617 mouse cDNA clones (Twin Chip Mouse-7.4K). A total of 40 genes are involved in the areas of 7 categories relating to growth, cell cycle, apoptosis, signal transduction, transcription, immune system, and stress. There are genes associated with the aging brain in some of the areas of the 7 categories. Firstly, genetic mutations in genes lead to disease. It was demonstrated that mutations of the *Harp* gene are responsible for deafness in the Jackson shaker 2 (js) mutant mice and in humans (28). The variation in the histamine H3 receptor (HRH3) in patients with Shy-Drager syndrome is also related to the etiology of the illness due to altered norepinephrine release (29). Moreover, a mutation in the *Mpz* genes has been found in patients with congenital hypomyelinating neuropathy (CHN), and *GNGT2* is the site of a mutation responsible for inherited retinal degeneration (30,31). In addition, mice deficient in TYK2 developed Abelson-induced B lymphoid leukemia lymphoma, and in *Rag1*$^{-/-}$ mutants, stage-specific increases were observed in the proliferation profile of fetal thymocyte compared to wild-type fetal thymocyte (32,33). Secondly, the identification of specific genes provides further insight into the understanding of the features of cell cycle, cell proliferation, and apoptosis. Human geminin (Gmnn) ensures basal levels of CDT1 during the S phase and its accumulation during mitosis (34). It was also demonstrated that centrin 2 (*Cetn2*) gene coding for the calcium-binding protein localizes in the region immediately surrounding the centrioles in the centrosome, which plays a diverse role throughout the cellular mitotic cell and in postmitotic cells (35). Cradd plays an important role in regulating apoptosis in mammalian cells, and EGLN3, a family of Egl-Nine (EGLN), is the human orthologue of rat Sm-20 that is a novel family of prolyl hydroxylase, which include a growth-responsive and cell-death-related protein (Sm-20) in mammals (36,37). Moreover, Mitsugumin 29 (MG29) serves an essential role in muscle Ca^{2+} signaling, which has been shown to regulate the process of cell proliferation and apoptosis (38). In addition, human cytoplasmic gelsolin inhibits Aß peptide-induced cell death

ally differentiated rat PC-12 cells (39). In the *Cdc25A* expression displayed increased immunoreactivity with the mitotic phosphoepitope-specific antibody and was correlated with proliferating cell nuclear antigen labeling index (40,41). Thirdly, the identified genes are related to embryogenesis, differentiation, and development. It has been demonstrated that Asxl1 is implicated in embryogenesis and carcinogenesis due to the transcriptional regulation of target genes through histone modification and chromatin remodeling, and Pcp2, known as L7, plays an important role in controlling the development and/or motor control function of Purkinje cells (42,43). Also, an up-regulation of *TACC* occurs during the early differentiation of PC12 cells into neurons and in embryonic, postnatally developing, and adult mouse tissue (44,45). In addition, Ly75 (*DEC-205*) transcripts were significantly increased upon differentiation (46). Fourthly, of the identified genes, the levels of gene expression lead to altered phenotypes. Expression of Toll-like receptor (*TLR*) 4, a member of interleukin-1 receptor (IL-1R), was decreased in the brain and is critically required for sustained inflammation on CNS resident cells in the brain after systemic administration of LPS (47,48). Moreover, a high level of *Car8* expression appeared in the pyramidal and granular cells of the hippocampus, and expression of *Slc23a1* is required for transporting vitamin C into the brain (49,50). Recently, the expression of ADAMTS protein was shown to have a role in a variety of diseases including Alzheimer's disease (51). In addition, yeast sec15 expressed synaptotagmin II, which is a neurally expressed protein, thought to be involved in neurotransmitter release from neurons (52). Other genes listed may also be associated with the aging process, but it is unknown. *Adrb2* was expressed in rat microglial cells (53).

In conclusion, we provide evidence that the approach of cDNA microarray leads to 48 or 40 differentially regulated genes in transgenic and normal mice upon APPsw over-expression. In the future, it will be necessary to study the function of differentially expressed gene, which are targets for developing drugs, using pharmacoproteomics.

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