SPANDIDOS PUBLICATIONS

Characterization of changes in global gene expression in the brain of neuron-specific enolase/human Tau23 transgenic mice in response to overexpression of Tau protein

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Abstract. Tau is a neuronal phosphoprotein responsible for the formation of the neurofibrillary tangles in Alzheimer's disease. To characterize the changes in global gene expression in the brain of transgenic mice that overexpress human Tau23 protein in response to the increase of Tau23 phosphorylation, total RNA extracted from the hippocampus of 12-month-old transgenic and wild-type mice was converted to cDNA, labeled with biotin and hybridized to oligonucleotide microarrays. The microarray results were confirmed by real-time RT-PCR and Western blotting method. It was determined that 43 genes were up-regulated and 8 genes were down-regulated by Tau23 in transgenic mice compared to controls, based on the arbitrary difference in the 2-fold change. Among the up-regulated transcripts, those encoding for transporter and oxidoreductase were dramatically over-represented, followed by those related to regulatory molecule, cytoskeletal protein, signaling molecule, and extracellular matrix protein. Genes encoding for transcription factor, regulatory molecule, miscellaneous function, and chaperone were significantly reduced in the down-regulated group. The major genes in the up-regulated categories included Ecrg4, Folr1, Defb11, Aqp1 and Soctdc1. The major genes in the down-regulated categories were Ncor1, Gpm6a, and Hspd1. These results indicate that the microarray analysis identifies several gene functional groups and individual genes that respond to a sustained increase in Tau23 phosphorylation levels in the brain of transgenic mice. In addition, the results suggest the microarray test is a useful tool for increased understanding of the role of Tau23 protein in regulating neurodegenerative disorders.

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Introduction

Alzheimer's disease (AD) is a brain disorder that leads to severe problems in memory, thought, and behavior. Patients with AD have difficulties in career, lifestyle, and social life. Currently, 12 million people are affected with AD and the number is predicted to triple by 2050 world-wide (1,2). The common pathogenic figures of AD are senile plaques, neurofibrillary tangles (NFTs), dystrophic neurites, extensive neuronal loss, and gliosis (3). Currently, the most credible hypothesis regarding senile plaques is that they form by reversible aggregation of Aß in clusters (4,5). Aß peptides are generated by sequential cleavages of APP by ß- and γ-secretase (6,7). The increased Aß production and deposition in the brain may trigger neuronal dysfunction and death in AD. Furthermore, NFTs, a different pathological hallmark of AD, are composed of paired helical filament (PHF)-Tau (8). Tau is a neuronal phosphoprotein that has tubulin-polymerizing activities *in vitro* and establishes short cross-bridges between axonal microtubules (9,10). Also, this protein is regulated by various protein kinases and phosphatases (11). Among the kinases, cyclin-dependent kinase-5 (cdk5) and glycogen synthase-kinase 3ß (GSK3ß) are predominant Tau kinases in the brain (12-15).

Phosphorylated Tau has a reduced affinity for microtubules and a reduced ability to promote microtubule assembly (16-19). Hyperphosphorylated Tau protein becomes aggregated into toxic insoluble filaments and leads to cell death. In the adult brain, Tau is primarily located in axons, but hyperphos-

phorylated Tau is found in cell bodies and dendrites in the AD brain. Results from *in vitro* studies suggest that the degree of dementia correlates with the severity of Tau-based NFTs (11). Such observations suggest that the phosphorylation of Tau plays a significant role in regulating the dynamics of microtubule assembly/disassembly in neurons (9). Several transgenic (Tg) mice were developed by inducing the overexpression of the human Tau (hTau) gene (20,21). In particular, neuron-specific enolase (NSE)/hTau23 Tg mice were generated by microinjection of the fusion gene into the male pronucleus of fertilized embryos in order to study the mechanism of AD. This fusion gene was constructed by the hTau23 gene cloned under the control NSE promoter (22). In these Tg mice, the increase of Tau phosphorylation and PHFs induced by the overexpression of Tau at the age of 12-months was observed. For this reason, these Tg mice serve as a useful model to study the relationship between genes and the mechanisms of AD. In addition, this model is available for testing new drug candidates. However, there is no study in which genes have been regulated by Tau23 phosphorylation using the modifications developed in these Tg mice.

In this study, we characterized the changes in global gene expression in the brain of Tg overexpressing hTau23 gene in response to an increased phosphorylation level of Tau23 protein. A total of 51 genes were identified using microarray analysis in 12-month-old Tg mice compared to wild-type mice. Also, these genes involved various genes related with cell physiology such as transporter, defense/immunity protein, oxidoreductase and transcription factor. Furthermore, some of the genes with up- and down-regulation were validated by quantitative RT-PCR and Western blotting. From the analysis of our data, we identified several known genes that had not been linked to AD in the past. This study may help in the search for genes associated to AD and will help with understanding the prevention, clinical diagnosis, and treatment of neurodegeneration.

Materials and methods

Animals. We used Tg mice that overexpressed the hTau23 gene, which were developed in our laboratory by microinjecting the fusion gene into the male pronucleus of fertilized embryos. Embryos were obtained by crossing C57BL/6 (female) mice with DBA2 (male) mice (22). All pedigrees were hemizygous for the implanted transgene. In order to determine the gene expression profiles, we used 12-month-old NSE/hTau23 mice (n=6) and wild-type mice (n=6) for purification of total RNA from brain. All mice were handled in an accredited Korea FDA animal facility in accordance with AAALAC International Animal Care policy (Accredited Unite-Korea Food and Drug Administration, Unit Number-000996). All experiments and protocols were approved by the Institutional Animal Care and Use Committee. The mice were housed in cages under a strict light cycle (lights on at 06:00 h and off at 18:00 h). All mice were given a standard irradiated chow diet (Purina Mills Inc., Korea) *ad libitum*, and were maintained in a specific pathogen-free state.

Western blotting. The proteins that were prepared from the hippocampuses of the Tg and wild-type mice were separated by electrophoresis in a 12% SDS-PAGE gel for 2 h at 40 Vol. Each membrane was incubated separately with the primary, anti-Tau (sc-5587, rabbit polyclonal, 1:2,500; Santa Cruz Biotechnology) at 37˚C for 2 h and anti-phospho-Tau (Thr^{231}, rabbit polyclonal, 1:500, Sigma), anti-Aqp1 (sc-25287, mouse monoclonal, 1:500, Santa Cruz Biotechnology), and anti-Folr 1 (sc-28997, rabbit polyclonal, 1:500, Santa Cruz Biotechnology) at 4˚C overnight. The membranes were washed with washing buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 0.05% Tween-20) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000) or anti-mouse IgG (1:5,000) at room temperature for 2 h. The membrane blots were developed using a Chemiluminescence Reagent Plus kit (ECL Plus; Amersham Biosciences).

RNA isolation. The hippocampuses from 12-month-old Tg and wild-type mice were used as the source of isolating total RNA through the use of Trizol (Invitrogen Life Technologies, Carlsbad, USA), then purified using RNeasy columns (Qiagen, Valencia, USA). Both were performed according to the manufacturer's protocol. First, 30 mg of frozen hippocampuses were lysed though the use of lysis buffer and homogenized using a rotor-stator homogenizer. Next, 70% ethanol was added to the homogenate, and samples were then applied to an RNeasy minispin column for total RNA to bind to the resin in the column. Total RNA was eluted in the RNase-free-water. After processing with DNase digestion, clean-up procedures, RNA samples were quantified, aliquoted, and stored at -80˚C until use. For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis, OD 260/280 ratio, and analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA).

Labeling and purification. Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, USA). To produce biotinylated cRNA according to the manufacturer's instructions, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo(dT) primer. A second-strand of cDNA was synthesized, transcribed *in vitro*, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA).

Hybridization and data export. We hybridized 750 ng of labeled cRNA samples to each mouse-6 expression bead array for 16-18 h at 58˚C, according to the manufacturer's instructions (Illumina, Inc., San Diego, USA). The array signal was detected using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned using an Illumina Bead Array Reader Confocal Scanner (BeadStation 500GXDW; Illumina, Inc.) according to the manufacturer's instructions. Array data export processing and analysis was performed using Illumina BeadStudio.

Raw data preparation. The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. We extracted the raw data using the software provided by the manufacturer (BeadStudio v.3.1). Array data were filtered by

value set at <0.05 (similar to signal to noise) in at samples. We applied a filtering criterion for data analysis; higher signal value was required to obtain a detection of P<0.05. Selected gene signal value was transformed by logarithm and normalized by Quantile method to remove systemic bias. Data reliability was measured by scatter plot method. Correlation coefficients (Rs) of normalized samples were revealed as ranging from 0.95 to 0.99, which indicates that systematic variation of non-biological origin was removed. The comparative analysis between transgenic and non-transgenic mouse samples were carried out using the t-test (|fold|> or <2) and the adjusted Benjamini-Hochberg FDR (false discovery rate) P-value (<0.05) (23). We performed volcano plots and hierarchical cluster analysis using complete linkage and Euclidean distance as measures of similarity. Time-dependent profiling was achieved by k-means cluster analysis (k, 9, Euclidean distance, complete linkage). All data analysis and visualization of differentially expressed genes were conducted using ArrayAssist® (Stratagene, La Jolla, USA). Biological pathway and ontology-based analysis were performed by using the Protein Analysis Through Evolutionary Relationships (PANTHER) database (http://www. pantherdb. org).

Quantitative real-time RT-PCR. cDNA was produced using the Superscript™ II RT-PCR System (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations for oligo (dT) 20-primed cDNA-synthesis. cDNA synthesis was performed on 500 ng of RNA, at 42˚C. Finally, cDNA samples were diluted 2-fold in water before QPCR analysis. PCR was performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, CA, USA), and performed in 384-well microtiter plates using a final volume of 10 μl. Optimum reaction conditions were obtained with 5 μl of Universal Master Mix (Applied Biosystems), which contained dNUTPs, $MgCl_2$, reaction buffer, Ampli Taq Gold, 90 nM of primer(s), and 250 nM fluorescence-labeled TaqMan probe. Finally, 2 μl template cDNA was added to the reaction mixture. The 6 primer/TaqMan probe combinations were designed from sequences from the NCBI public database. Amplifications were performed starting with a 10 min template denaturation step at 95˚C, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. All samples were amplified in triplicate, data were analyzed with sequence detector software (Applied Biosystems). After normalization with GAPDH, we analyzed the expression level of genes in experimental groups using the comparative method (ΔCt). Then, these data that analyzed the above results were also compared with data obtained from microarray in order to verify the variation between the two methods.

Statistical analysis. In the microarray analyses, significance between wild-type mice and Tg mice were performed using a t-test. Also, one-way ANOVA test (SPSS for Windows, Release 10.10, Standard Version, Chicago, USA) were performed to determine the variance and significance between wild-type mice and Tg mice on the level of specific protein expression. All values are represented as the mean ±standard deviation (SD), with the mean difference between Tg mice considered statistically significant at P<0.05.

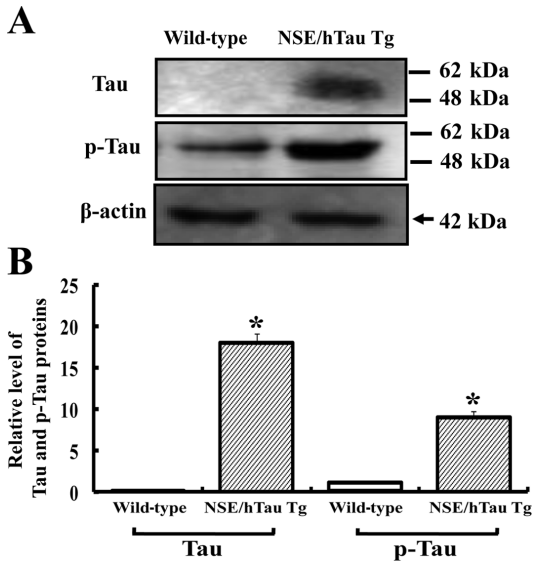

Figure 1. Expression level of total and phosphate-form Tau23 in wild-type and NSE/hTau23 Tg mice. The hippocampal tissues of 12-month-old wild-type mice and Tg mice were pooled, respectively, prior to protein analysis. Tau overexpression and its phosphorylation were examined by Western blotting using anti-Tau and phospho-Tau ($pThr^{231}$) antibodies. Data represent the mean ±SD from three replicates. *P<0.05, significant level compared with the Tg mice.

Results

Change of Tau expression and phosphorylation in NSE/hTau23 Tg mice. It was reported that the expression level of Tau protein was significantly higher in the NSE/hTau23 Tg than in wild-type mice (22). Therefore, first we examined whether hTau23 gene was successfully expressed and phosphorylated in the Tg mice used in this study. Expression level of hTau23 protein and p-hTau23 were detected in the samples of hippocampuses from 12-month-old mice. As shown in Fig. 1, the Tg mice showed a higher level of hTau proteins than wild-type mice. Furthermore, the level of p-Tau protein was significantly increased in the hippocampuses of Tg mice compared to that of wild-type mice, although this antibody had a cross-activity for mice protein. These results suggested that the hippocampus region of Tg mice showed a higher level of total hTau and p-hTau protein than wild-type mice, respectively.

Image of the gene expression profile on cDNA microarray. A microarray analysis plot of the microarray gene expression data is shown in Fig. 2. The location of average gene expression values along the first diagonal showed a non-systematic bias between the wild-type and Tg groups. A higher variance was observed at the higher compared to lower expression levels. A fairly considerable number of genes were also expressed at elevated or at reduced levels in a Tg mouse hippocampus compared to the wild-type mice hippocampus. However, expression levels of most genes showed no difference between the Tg and wild-type hippocampus.

Filtering and selection for differentially-expressed genes. To identify the genetic profile of particular genes involved in response to phosphorylation of Tau23 protein, total RNA of each hippocampal tissue was successfully hybridized to the

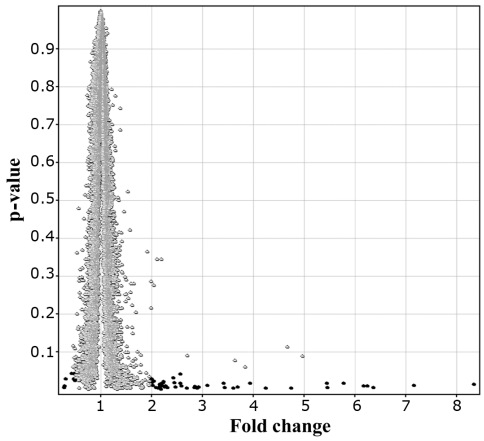

Figure 2. Volcano plots. Each point represents the average expression value for the same gene from the wild-type and Tg mice. The vertical axis represents the P-value and the horizontal axis represents the fold change. Black points represent the 51 significant genes that display biological importance and statistical significance.

Table I. Selection of differentially expressed genes.

Categories	Number of transcripts
Total	24,886
Filtered gene	12,072
|fold|≥2	51

Among a total of 24,886 individual mouse transcripts, 51 transcripts were selected as differentially expressed on a statistically significant basis in comparison to the control mice because the expression levels showed a fold change of ≥2 with a P <0.05.

Mouse-6 expression Beadchip microarrays containing 24,886 probes. A total of 12,072 genes were detected as differentially expressed genes in Tg mice compared to wild-type mice. Of these genes, further analysis was performed based on the arbitrary difference in the 2-fold change with a t-test, P<0.05. It was revealed that 51 genes were up-regulated and down-regulated in the Tg mice compared to the wild-type mice (Table I). These results suggested that the novel genes were successfully identified by the microarray analysis under the condition of Tau23 overexpression.

Ontology categories of Tau23-regulated gene expression. To analyze the ontology classification of the Tau23-regulated gene expression in the hippocampus of Tg mice, the gene lists were categorized into functional clustering according to the Gene Ontology classification using the PANTHER/X ontology on-line software; the results are presented in Table II. PANTHER/X ontology identified 15 GO categories showing significantly elevated expressions in the list of up-regulated transcripts (Table IIA). As presented in Table II, the largest number of transcripts turned out to be associated with transporter proteins and oxidoreductase proteins. From the list of down-regulated transcripts, PANTHER/X ontology identified 5 markedly overexpressed GO categories (Table IIB). In this group, the transcripts of those genes located on the transcription factor were especially highly expressed, followed by those involved in regulatory molecules, miscellaneous function, and chaperone. These results suggest that Tau23 protein regulates the expression of genes related to transporter, oxidoreductase activity, and transcription factor for gene expression in the hippocampuses of Tg mice.

Table II. Gene ontology categories. Gene ontology was classified by using PANTHER database categories.

A, Up-regulated genes involved 14 categories and 1 unclassified group at the level of significance.

P-value	GO category	Number of transcripts
0.0119	Transporter	5
0.0123	Oxidoreductase	5
0.0092	Regulatory molecule	3
0.0210	Cytoskeletal protein	3
0.0097	Signaling molecule	2
0.0078	Extracellular matrix protein	2
0.0081	Hydrolase	2
0.0043	Cell junction protein	2
0.0196	Ion channel	2
0.0050	Defense/immunity protein	2
0.0118	Transcription factor	2
0.0057	Receptor	1
0.0074	Miscellaneous function	1
0.0080	Transferase	1
0.0160	Molecular function unclassified	10

B, Down-regulated genes involved 4 categories and 1 unclassified group at the level of significance.

P-value	GO category	Number of transcripts
0.0191	Transcription factor	2
0.0428	Regulatory molecule	1
0.0295	Miscellaneous function	1
0.0426	Chaperone	1
0.0195	Molecular function unclassified	3

Genes with the highest expression level of up- and down-regulation. In order to characterize the genes showing the highest overexpression, those with at least a 2-fold increase were selected from the 43 up-regulated genes and 8 down-regulated genes. As shown in Table III, the highest increase was in transcripts Ecrg4 (8.35-fold) identified as a cancer-related gene, followed by Folr1, Defb11, Aqp1, Sostdc1, and Prlr. The various transporters and immunity proteins were elevated by the regulation of Tau23 protein in the hippocampuses of Tg mice. In addition, 8 genes with the highest fold change were significantly decreased (Table IV). Of these genes, RIKEN cDNA 9630050M13 gene which has unidentified functions, was the most most dramatically decreased, followed by Ncor1, Gpm6a, Hspd1, Rab7 and Rnfl4. These results indicate that Tau23 protein is closely

Genes (43) with the highest folding increase among those up-regulated in the hippocampus of NSE/hTau23 Tg mice.

Gene symbol	Gene name	Accession No.	Fold of change	P-value	GO category
Ecrg4	esophageal cancer related gene 4	NM_024283	8.35	0.0140	
Folr1	folate receptor 1	NM_008034	7.18	0.0110	Transporter
Defb11	defensin ß 11	NM_139221	6.37	0.0057	Defense/immunity protein
1600023A02Rik		NM_026323	6.24	0.0098	regulatory molecule
Aqp1	aquaporin 1	NM_007472	6.17	0.0106	Transporter
Sostdc1	sclerostin domain containing 1	NM_025312	5.78	0.0172	
Prlr	prolactin receptor	NM_011169	5.46	0.0057	Receptor
Clic6	chloride intracellular channel 6	NM_172469	5.46	0.0178	Ion channel
Cldn2	claudin 2	NM_016675	4.75	0.0043	Cell junction protein
Cox8b	cytochrome c oxidase, subunit VIIIb	NM_007751	4.23	0.0050	Oxidoreductase
Calml4	calmodulin-like 4	NM_138304	3.95	0.0177	
Rdh5	retinol dehydrogenase 5	NM_134006	3.68	0.0069	Oxidoreductase
Lbp	lipopolysaccharide binding protein	NM_008489	3.60	0.0043	Defense/immunity protein
Kl	klotho	NM_013823	3.43	0.0063	Hydrolase
Otx2	orthodenticle homolog 2 (*Drosophila*)	NM_144841	3.41	0.0173	Transcription factor
Col8a2	procollagen, type VIII, α 2	NM_199473	3.09	0.0113	Extracellular matrix protein
1600023A02Rik	RIKEN cDNA 1600023A02 gene (1600023A02Rik), mRNA	NM_026323	2.93	0.0069	regulatory molecule
Cldn1	claudin 1	NM_016674	2.89	0.0043	Cell junction protein
	RIKEN cDNA 2810046M22 gene	NM_026621	2.85	0.0085	
1110059M19Rik	RIKEN cDNA 1110059M19 gene	XM_135842	2.84	0.0066	
Col8a1	procollagen, type VIII, α 1	NM_007739	2.70	0.0043	Extracellular matrix protein
Sulf1	sulfatase 1	NM_172294	2.59	0.0099	Hydrolase
Mia1		NM_019394	2.56	0.0177	
Dncl2b	dynein, cytoplasmic, light chain 2B	NM_029297	2.56	0.0416	Cytoskeletal protein
Igfbp2	insulin-like growth factor binding protein 2	NM_008342	2.46	0.0074	Miscellaneous function
Tuba6	tubulin, alpha 6	XM_147357	2.45	0.0193	Cytoskeletal protein
AI427515	expressed sequence AI427515	NM_173016	2.42	0.0321	Oxidoreductase
Msx1	homeo box, msh-like 1	NM_010835	2.31	0.0063	Transcription factor
Cdkn1c	cyclin-dependent kinase inhibitor 1C (P57)	NM_009876	2.27	0.0109	regulatory molecule
Sema3b	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	NM_009153	2.26	0.0080	Signaling molecule
Trpm3	transient receptor potential cation channel, subfamily M, member 3	NM_177341	2.23	0.0213	Ion channel
Slc39a4	solute carrier family 39 (zinc transporter), member 4	NM_028064	2.22	0.0193	Transporter
2410146L05Rik	RIKEN cDNA 2410146L05 gene	NM_026480	2.22	0.0099	
Tekt1	tektin 1	NM_011569	2.18	0.0022	Cytoskeletal protein
Tsga2	testis specific gene A2	NM_025290	2.18	0.0163	
2310016C16Rik	RIKEN cDNA 2310016C16 gene	NM_027127	2.17	0.0117	Oxidoreductase
Hemk1		NM_133984	2.16	0.0080	Transferase

Table III. Continued.

Gene symbol	Gene name	Accession No.	Fold of change	P-value	GO category
Slc4a2	solute carrier family 4 (anion exchanger), member 2	NM_009207	2.16	0.0142	Transporter
Aqp1	aquaporin 1	NM_007472	2.12	0.0043	Transporter
2310016C16Rik	RIKEN cDNA 2310016C16 gene	NM_027127	2.08	0.0057	Oxidoreductase
1700009P17Rik	RIKEN cDNA 1700009P17 gene	NM_029301	2.04	0.0237	
Igf2	insulin-like growth factor 2	NM_010514	2.03	0.0114	Signaling molecule
BC013481	cDNA sequence BC013481	NM_178446	2.01	0.0287	

Table IV. Genes (8) with the highest folding decrease among those down-regulated from the hippocampus of NSE/hTau23 Tg mice.

Gene symbol	Gene name	Accession No.	Fold of change	P-value	GO category
9630050M13Rik	RIKEN cDNA 9630050M13 gene	XM_194000	-3.72	0.0057	
Ncor1	nuclear receptor co-repressor 1	NM_011308	-3.50	0.0099	Transcription factor
Gpm6a	glycoprotein m6a	NM_153581	-3.27	0.0295	Miscellaneous function
Hspd1	heat shock protein 1 (chaperonin)	NM_010477	-2.38	0.0426	Chaperone
C030032C09Rik	RIKEN cDNA C030032C09 gene	NM_181398	-2.14	0.0283	
Rab7	RAB7, member RAS oncogene family	NM_009005	-2.08	0.0428	Regulatory molecule
5830457O10Rik	RIKEN cDNA 5830457O10 gene	NM_145412	-2.05	0.0246	
Rnf14	ring finger protein 14	NM_020012	-2.03	0.0283	Transcription factor

Table V. Comparison of expression ratios of selected genes from the hippocampus of NSE/hTAu23 Tg and wild-type Tg mice, as determined by real-time PCR and microarray analysis.

Gene symbol	Accession No.	Microarray fold change	Q-PCR fold change
Lbp	NM_008489	3.60	4.81
Folr1	NM_008034	7.18	12.27
Aqp1	NM_007472	6.17	13.94
Ncor1	NM_011308	-3.50	-1.03
Gpm6a	NM_153581	-3.27	-1.01

involved in the regulation of genes that function as transporters, defense proteins, regulatory molecules, and transcription factors under the condition of a Tau23 abnormal phosphorylation level.

Validation of microarray data. To validate the reliability of the results obtained from the microarray analysis, we performed real-time RT-PCR. We selected candidate genes according to the following criteria: i) genes showing a high magnitude in fold change, ii) genes annotated with function, and iii) genes linked to neuronal diseases. Accordingly, five up-regulated genes (Lbp, Folr1, and Aqp1) and two down-regulated genes (Ncor1 and Gpm6a) were selected and analyzed. When comparing the results of the microarray data and that of the real-time RT-PCR, the results of PCR analysis were further increased in the 3 up-regulated genes, although pattern increase was very similar in the two methods. However, the expression

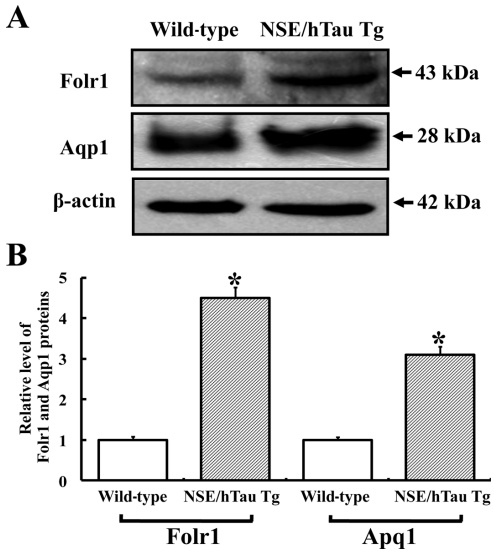

Figure 3. Change of Folr 1 and Aqp 1 protein level in wild-type and NSE/hTau23 Tg mice. The hippocampal tissues of 12-month-old wild-type mice and Tg mice were pooled, respectively, prior to protein analysis. Folr 1 and Aqp 1 expression were examined by Western blotting using anti-Folr 1 and Aqp1 antibodies. The data represents the mean ±SD from three replicates. *P<0.05, significant level compared with Tg mice.

regulated genes was decreased to a lesser extent in analysis compared to the microarray data (Table V). Furthermore, to investigate correlation between transcriptional and protein levels, we analyzed protein expression levels by Western blotting. Folr1 and Aqp1 were chosen, based on the greatest changes (12- and 14-fold increases, respectively) among the candidates. As shown in Fig. 3, we observed the apparent up-regulation of Folr1 and Aqp1 expression in protein level. Therefore, this result corroborates the data from our microarray analysis.

Discussion

In normal human nerve cells, Tau is a normally soluble protein essential for maintaining the shape and structural integrity. Also, this protein plays an important role in transportation between cellular compartments by association with neuronal axons. Under pathological conditions, Tau is excessively phosphorylated, which destabilizes microtubules. Several Tau animal models have been developed by introducing the wild-type/mutant Tau gene or altering the Tau-transgene promoter. Although none have completely reproduced the disease process, animal models are widely accepted as an excellent tool for studying the disease mechanism and screening novel drugs.

Here we found several interesting genes related to AD mechanism and pathogenesis. The Folr gene encodes folate receptor α (FRα), which has the ability to bind folate (24). RT-PCR analysis, using RNA extracted from the fibroblast of AD, showed that, from cultured fibroblasts of AD, FRα mRNA was significantly higher in AD fibroblasts than in controls, which suggests that higher folate binding in AD fibroblast is due to an enhanced expression of FRα (25). Its up-regulation is very similar to that determined by this microarray study. Therefore, this gene may serve as a biomarker for AD diagnosis. Aquaporins (AQPs) are a family of transmembrane proteins that act as water selective channels (26). In the brain, AQP1 is found in the apical pole of the choroidal plexus of epithelial cells and ependimocytes (27,28). A possible relationship between the AQP increase and spongiform degeneration has been suggested by AQP1 increase in Creuzfelt-Jakob disease (CJD) patients and in Bovine spongiform encephalopathy (BSE) found in infected mice (29). Interestingly, Western blotting studies have shown a significant increase in the level of *aqp1* in the frontal cortex in sporadic AD cases (30). In our study, *aqp1* was found, for the first time, to be expressed highly in the hippocampus in the model mice for AD. It may be expected that the tau overexpression alters AQP1 level, which disrupts water and ion homeostasis in the cell, and may induce the neurodegenerative mechanism of AD. Claudin is a multigene family member protein that is involved in the formation of tight junctions in various tissues (31,32). A highly sensitive nested RT-PCR analysis showed that from claudin-2 to -8 mRNA was present in the rat hippocampus and in frontal cortex tissues (33). Although individual claudin may be responsible for the establishment or maintenance of tissue architecture, specific physiological roles for the particular claudins are still unknown. The blood-brain barrier (BBB) has been considered to be intact during neurodegenerative diseases such as AD and Parkinson's disease (PD), however, recent evidence argues otherwise. Endothelial cells exposed to 20 μM of $A\beta_{1-42}$ altered the expression pattern of tight junction proteins, occludin, claudin-1, and ZO-2 (34,35). In the present study, our results reveal a 4.75-fold increase of claudin-2 mRNA level and 2.89-fold increase of claudin-1 mRNA in the Tg mice overexpressing Tau protein. These results support the hypothesis that tight junction components in BBB dysfunction induce neurodegenerative disease progression. It was also recently suggested that BBB development is controlled by Wnt/ß-catenin signaling (36-38). Liebner *et al* suggested that Wnt/ß-catenin signaling drives claudin expression, based on results from experiments using mice expressing both loss and gain of function mutant of ß-catenin. Previously, the hTau23 Tg mice exhibited high levels of GSK3ß phosphorylation with advanced age, resulting in increases in the phosphorylations of tau and ß-catenin. Thus, it is possible that claudin genes showing altered expression levels in the transgenic mice are regulated by phosphorylation of these proteins directly or indirectly.

The nuclear receptor corepressor (N-CoR1) is a co-regulator required for mediation of active repression by specific nuclear receptors and several additional classes of transcription factors (39,40). *In situ* hybridization and dual-label immunofluorescence studies have shown that N-CoR1 is expressed ubiquitously in the brain tissue of young adult male rats (41). Our results reveal that overexpression of human wild-type Tau reduced *N-CoR1* transcript, but this finding appears to be in conflict with previous microarray data using SH-SY5Y neuroblastoma cells expressing P301L mutant Tau. Hoerndli *et al* (42) have suggested that N-CoR1 was modestly up-regulated (1.58-fold) when $A\beta_{42}$ treatment was combined with P301L tau expression. Despite the fact that N-CoR1 was identified years ago as prominently expressed in the brain, little is known about its biological involvement in brain disorders or in AD. Glycoprotein M6a (Gpm6a, M6a) is a neuronal membrane protein (43,44). Alfonso *et al* (45) have shown that M6a plays an important role in neurite/filopodium outgrowth and synapse formation. They found the *M6a* gene was down-regulated in the hippocampus of socially and physically stressed animals. In the hippocampus of AD patients, M6a expression has been shown to be down-regulated (46). Here, we have provided compelling evidence that the *M6a* gene is decreased in the transgenic mouse, therefore, these data support the notion that chronic stress by Tau pathology is related with M6a protein dysfunction and AD progression.

There is little overlap between the gene profiles reported in this study and those from previously-reported microarray-based studies in AD brain tissues. This could be due to diversity and complexity in AD pathogenesis in humans, compared to our system which overexpresses a single gene. In addition, subtle methodological differences, such as statistical methods in normalization and filtering, and microarray platforms, might be responsible for disconcordance between the mouse and human microarray data. Since AD pathogenesis is still incompletely understood, our results should be valuable in interpreting data from future studies on AD pathogenesis in human as well as animal models.

In conclusion, we identified and validated differential gene expression due to the overexpression of human Tau protein. The hippocampal regions from 12-month-old Tg and wild-type mice were used. The hippocampus is selected because this tissue is

closely linked to the declining cognitive performance in AD patients. The present study identified 51 differentially expressed genes associated with Tau pathology in the humanized AD model mice. This was confirmed by performing quantitative reverse PCR and Western blotting. Thus, these genes may contribute to the development of new therapeutic targets.

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