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Identification of genes associated with age-related memory impairment in rats

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Abstract: A large body of evidence indicates that memory impairment is associated with normal aging. Interestingly, some older individuals do not show any memory loss. To understand the molecular mechanisms of the age-related memory disorder, gene expression profiles of hippocampus and entorhinal cortex from 24-month-old memory-impaired and memory-unimpaired rats that were divided based on their performance in Morris water maze were examined using high-density DNA microarrays. The results demonstrated that 47 genes in the hippocampus and 37 genes in the entorhinal cortex showed dynamic changes in their expression levels. Surprisingly, the overall patterns of gene expression changes in these two brain regions were significantly different. Nevertheless, a number of key genes involved in structure organization, neurotransmission, signaling transduction, transcription, immunity and oxidative signaling were differently expressed in both brain regions. These genes and signal pathways may play essential roles in the regulation of memory. Our results provided important information for understanding the molecular mechanism of age-related memory impairment.

Key words: genes; memory impairment; aging; neurotransmission

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利用基因芯片检测大鼠衰老相关的记忆障碍的调控基因

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摘要: 许多证据表明, 正常衰老过程伴随着记忆力的衰退. 但有些动物却不表现出这种年龄相关的记忆障碍. 为了检测其中的分子机制, 将 24 个月的老年大鼠按在水迷宫中的行为表现分成记忆损伤组和记忆未损伤组, 分别取海马和内嗅皮层进行基因芯片检测. 结果显示, 在海马和内嗅皮层中分别有 47 和 37 个基因的表达发生了显著变化. 但两个脑区的基因表达变化模式却有明显的不同. 对差异表达的基因进行功能分析, 主要是与结构组织、突触传递、信号转

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导、转录及免疫和氧化信号相关的基因. 这些基因和信号通路可能在记忆的调控过程中起重要作用. 该结果为进一步揭示衰老相关的记忆衰退过程的分子机制提供了重要的信息.

关键词: 基因; 记忆障碍; 衰老; 突触传递

0 Introduction

Memory declines with aging in many animals including fruit fly[1,2], mouse[1,2], rat[3,4], monkey[5,6] and human[7,8]. Although these organisms show age-dependent memory deterioration, some older animals do not show significant memory deficits[9,10]. The molecular mechanism of age-dependent memory impairment remains to be elucidated. It is well known that memory formation and storage need new gene expression[11,12]. Many genes involved in the regulation of learning and memory have been identified[13,14]. Furthermore, gene expression profiling of the brain in different animals demonstrated that many genes and signal pathways were involved in learning and memory processes[15,16]. Several groups have used microarray technique to study the underlying mechanism of age-dependent memory decline. Blalock *et al* showed that alterations of genes involved in neuronal activity and metabolism inhibited neurite growth that, in turn, triggered a demyelination process and an inflammatory cascade in aged brain. These changes eventually impaired memory[17]. Recently, Verbitsky *et al* analyzed the hippocampal gene expression profile in 2-month-old young mice and 15-month-old middle-aged mice. They found altered expression in genes related to synaptic plasticity, inflammation, protein processing, and oxidative stress between these two groups of mice[18]. Since middle-aged mice only show a mild memory deficit, these changes may not explain age-related memory deficit.

Extensive studies indicated that structure changes and synaptic dysfunction of hippocampus were correlated with age-dependent memory deficits[19,20]. Entorhinal cortex is another important brain region involved in memory. In this report, we have analyzed the gene expression profiling from hippocampus and entorhinal cortex in memory-impaired and memory-unimpaired 24-month-old rats.

1 Experimental procedures

1.1 Animals and treatment

Female Fischer 344 rats at the age of 24-month were divided into two groups, memory unimpaired and impaired, based on their performance in the Morris water maze task. The animal care procedures and protocols of water maze were the same as previously described[21]. To divide the aged animals into memory-impaired and memory-unimpaired, the percentage of total distance swum in the target quadrant during probe trial was calculated. If an aged rat performed within the range of young rats, it was considered memory-unimpaired. Otherwise, if the rat fell below the range of young rats, it was considered memory-impaired (The cutoff was 30.8%). Five animals from each group were rapidly decapitated. Hippocampus and entorhinal

cortex were collected and stored at −80 ℃ before use. The tissues were provided by Dr. Mark Tuszynski from University of California.

1.2 Gene expression analysis

Total RNA was purified with Trizol reagent (Invitrogen). RNA samples were measured on Agilent 2 100 bioanalyzer. RNA with RIN number higher than 8 was labeled and hybridized to high-density oligonucleotide microarray (GeneChip, Affymetrix, Santa Clara, CA) as previously described[22]. Data were analyzed with the Affymetrix GeneChip Expression Analysis Software. To ensure the reliability of the data, we conducted hybridization experiments in duplicates. Two independent mRNA samples from the same tissue were hybridized to two sets of duplicate microarrays. Gene expression change in both arrays was collected for further analysis.

1.3 Real-time PCR

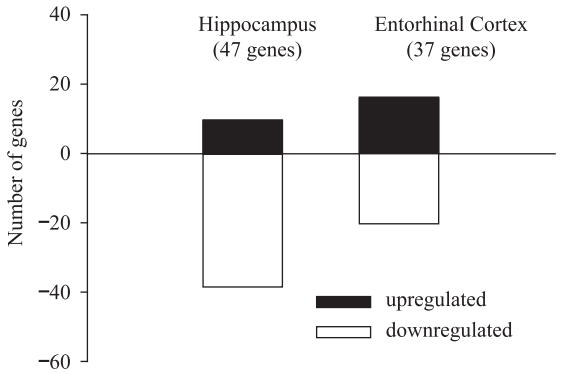
Some selected genes were validated by TaqMan Real-time RT-PCR (ABI 7 700 Sequence Detection system). Total RNA isolated from hippocampus or entorhinal cortex was reverse-transcribed using the SuperScript First-Strand Synthesis System (INVITROGEN, Carlsbad, CA). The sequences of primers and probes of the tested genes and GAPDH, an endogenous control, were listed in supplementary material.The PCR reaction was performed using 50 μL of total reaction mixture volume and 2 μL of cDNA reaction products at 1 cycle of 55 ℃ for 1 min, 1 cycle of 95 ℃ for 10 min, and 40 cycles of 95 ℃ for 30 s and 55 ℃ for 1 min. All the probes (Perkin-Elmer) were labeled with the fluorescent dyes 6-carboxyfluorescein (FAM) and 6-carboxy-tetramethyl rhodamine (TAMRA). The fold change of the targets genes was calculated using the $2^{-\Delta\Delta Ct}$ methods.

2 Results

Aged memory-impaired and unimpaired rats were used for gene expression profiling analysis in the hippocampus and entorhinal cortex. Our results showed that expression levels of 47 and 37 genes were altered more than 2-fold in the hippocampus and entorhinal cortex, respectively (see Fig. 1). Surprisingly, among the changed genes, only seven of them were commonly regulated in the hippocampus and entorhinal cortex. Three of the seven genes were down-regulated in both brain regions, while the other four genes showed opposite regulation in their expression levels (see Tab. 1). These results suggested that different molecular events might take place in these two brain regions in the age-associated memory disorder process.

We further confirmed the gene expression changes using quantitative RT-PCR experiment. Nine up-regulated genes and five down-regulated genes were chosen for the quantitative PCR experiment. Our results demonstrated that the expression changes from these genes could be validated by quantitative RT-PCR (see Tab. 2).

Classification based on their physiological functions demonstrated that genes involved in structure organization, neurotransmission and signaling transduction, transcription, immunity and oxidative signaling were altered in both hippocampus and entorhinal cortex in memory-impaired rats compared with unimpaired rats (see Fig. 2, Tab. 3 and Tab. 4).



Note: Black bars and white bars represent the number of genes up-regulated and down-regulated, respectively.

Fig. 1 Gene expression change in memory-impaired rats compared with memory un-impaired rats

Tab. 1 Genes commonly regulated in both entorhinal cortex and hippocampus of the memory-impaired rats

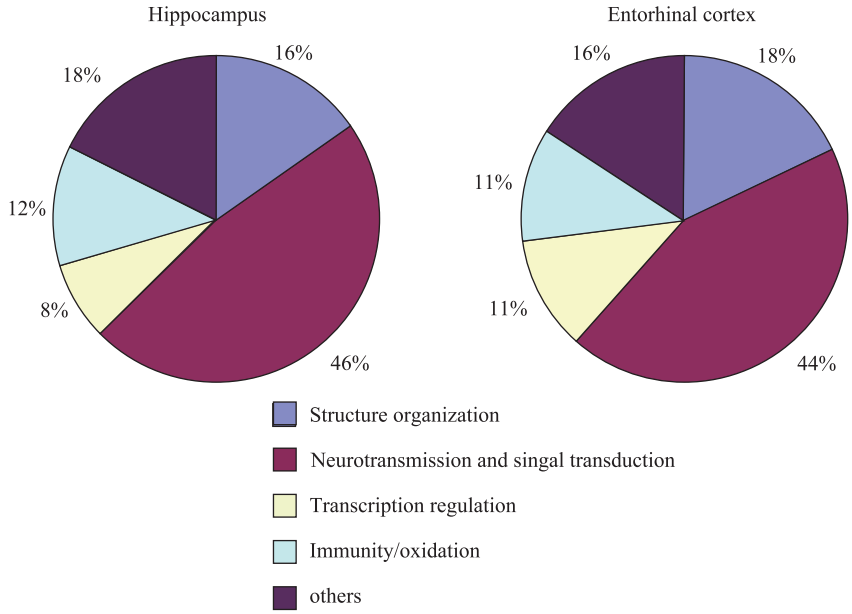
Accession number	Gene name	Fold change*	
		Hippocampus	Entohinal cortex
M15562	MHC class II RT1.u-D-alpha chain	−2.1	−2.7
X13044	MHC-associated invariant chain gamma(cd74)	−2.2	−16.3
AA925762	Myristoylated alanine rich protein kinase C substrate	−2.8	−2
V01244	Prolactin	−5.2	9.1
AA945169	Transthyretin	−9.9	5.5
AA894148	Apolipoprotein A-IV	−2.3	2.2
X62952	Vimentin	−6.1	3

Note: *Positive fold change values indicate an increase, and negative values indicate a decrease in gene expression of the memory-impaired rats compared to memory-unimpaired ones

Tab. 2 Validation of microarray-based expression profile by real-time RT-PCR

Accession number	Gene name	Fold change*	
		Real-time RT-PCR	Microarray
D83036	Msx-1 protein	−5.5	−2.4
AI012051	BAZ2B	−2.9	−2.0
Z46882	TOAD-64	4.6	14.0
V01244	Prolactin	12.9	9.1
AA894148	Apolipoprotein A-IV	3.8	2.2
X62952	Vimentin	4.2	3.0
AA945169	Transthyretin	3.9	5.5
AI102411	Brain-type clathrin	9.4	5.6
J04488	Prostaglandin D synthetase	−5.2	−4.8
L36664	Kininase II	−2.0	−3.0
AB017820	Klotho	−4.3	−2.4
AI171462	CD24	4.8	2.3
J02752	Acyl-coA oxidase	3.2	2.5
AA892801	Elongation factor 2	4.2	2.1

Note: *Positive fold change values indicate an increase, and negative values indicate a decrease in gene expression of the memory-impaired rats compared to memory-unimpaired ones



Note: Functional categorization was analyzed using MAPPFINDER/GENMAPP software; Percentages of genes whose expression levels were changed by age-dependent memory impairment were indicated

Fig. 2　Functional categories of genes whose expression levels altered in memory-impaired rats

Structure organization　A group of structure-related genes changed their expression levels in aged memory-impaired rats. In the hippocampus, the expression levels of six genes encoding structure organization or cytoskeleton were altered. Interestingly, all of these six genes were down-regulated. Similarly, the expression levels of eight structure-related genes were changed in entorhinal cortex of memory-impaired rats. Surprisingly, the expression of vimentin was decreased by 6.1-fold in the hippocampus, while it was up-regulated in the entorhinal cortex. In addition, the expression of microtubule-associated-protein 2 and 2c was down-regulated in entorhinal cortex of memory-deficient animals. Neurofilament-M (NF-M) and Neurofilament-L (NF-L), both of which are neuronal intermediate filament proteins and important components of the cytoskeleton, were up-regulated in entorhinal cortex.

Neurotransmission and signal transduction　This group of genes included a number of neurotransmitter transport proteins, ion channels, postsynaptic receptors and synaptic vesicle-associated proteins. Gamma-aminobutyric acid (GABA) receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and serotonin 1c receptor changed their expressions in the hippocampus or entorhinal cortex of memory-impaired rats. In addition, the expression levels of some genes that participate in synaptic vesicle trafficking and neurotransmitter release were altered. For example, SV2 related protein, an evolutionarily conserved synaptic vesicle protein[23], was up-regulated by 3.5-fold in the hippocampus. Several other synaptic proteins, such as complexin 2 (Cplx2), and PSD-95/SAP90-associated protein-3 changed their expression in the entorhinal cortex. A number of genes encoding signaling transduction proteins, such as protein kinases, phosphatases and other signaling molecules could be involved in age-related memory disorder. It was found that cyclin-dependent kinase 5 was up-regulated, while phosphodiesterase I and protein phosphatase 2C were down-regulated

in the hippocampus of memory-impaired rats. In the entorhinal cortex, the expression levels of calmodulin-binding protein (RC3), CaM-kinase II inhibitor alpha and protein kinase C type III were decreased, but the level of serum and glucocorticoid-inducible kinase (SGK) was increased. Furthermore, disabled 2 (Dab2), an essential component of transforming growth factor-β (TGF β) signaling pathway, and type III TGF-beta receptor showed a reduced expression in hippocampus of memory-impaired rats. We also found that homer 3, an adaptor protein regulating the interaction of group 1 metabotropic glutamate receptors with inositol trisphosphate receptors, was down-regulated about 3-fold in the entorhinal cortex. In addition, the expression level of inositol-1, 4, 5-triphosphate receptor was also reduced. It was interesting to note that Klotho, a hormone-like protein that is involved in the regulation of aging and longevity, was decreased by 2.4-fold in the hippocampus of memory-deficient rats. Brain-specific glycoprotein prostaglandin-D-synthase that participate in the isomerization of prostaglandin H2 (PGH2) to prostaglandin D2 (PGD2)[24] showed a 4.8-fold reduction in hippocampus of memory-impaired rats. In addition, genes involved in insulin-like growth factor (IGF) signaling, such as Igf-2, Igf-1, Igf binding protein-2 were down-regulated in the hippocampus or entorhinal cortex of memory-declined rats.

Tab. 3 Gene expression change in the hippocampus of memory-impaired rats

Classification	Accession number	Gene name	Fold change*
Structural	X62952	Vimentin	−6.1
organization	AA925762	Myristoylated alanine rich protein kinase C substrate	−4
Neurotransmission/	AA945169	Transthyretin	−9.9
signal transduction	AB016161	GABAB receptor 1d	−6.1
	M21410	5-hydroxytryptamine (serotonin) receptor 2 C	−4.8
	D28560	Phosphodiesterase I	−5.3
	S90449	Protein phosphatase 2 C	−2.4
	M80784	Type III TGF-beta receptor	−2.5
	AB017820	Klotho	−2.4
	J04488	Prostaglandin D synthetase	−4.8
	X17012	Insulin-like growth factor II	−19.1
	J04486	Insulin-like growth factor binding protein-2	−7.9
	V01244	Prolactin	−5.2
	L02121	Cyclin-dependent kinase 5	2.9
	AF060173	SV2 related protein (SVOP)	3.5
Transcription	U78102	Zinc finger protein krox-20/egr-2	−4.2
regulation	D83036	Msx-1 protein	−2.4
	AI012051	Bromodomain adjacent to zinc finger domain, 2B (BAZ2B)	−2
	U17607	Nuclear factor YC	9.6
Immunity/	X13044	MHC-associated invariant chain gamma(CD74)	−2.2
oxidation	M15562	MHC class II RT1.u-D-alpha chain	−2.1
	AA998683	Heat shock protein 27	−6.7
	X07365	Glutathione peroxidase	−2
	J02752	Acyl-coA oxidase	2.5

Note: *Positive fold change values indicate an increase, and negative values indicate a decrease in gene expression of the memory-impaired rats compared to memory-unimpaired ones

Transcription regulation We have found that some transcription factors changed

their expression levels in the hippocampus and entorhinal cortex of memory-deficient rats. Zinc finger protein early growth response protein 2 (egr-2), Msh homeobox 1 (Msx-1) protein and bromodomain adjacent to zinc finger domain, 2B (BAZ2B) were down-regulated, while nuclear transcription factor-Y gamma was up-regulated in the hippocampus of memory-impaired rats. In the entorhinal cortex, other transcription factors such as CTD-binding, SR-like protein, nuclease sensitive element binding protein and small unique nuclear receptor co-repressor (SUN-CoR) showed reduction of expression, while neurogenic differentiation 1 and nuclear receptor subfamily 4 increased their expression in memory-impaired rats.

Tab. 4　Gene expression change in the entorhinal cortex of memory-impaired rats

Classification	Accession number	Gene name	Fold change*
Structural organization	AA925762	Myristoylated alanine rich protein kinase C substrate	−2.8
	X53455	Microtubule-associated protein 2	−2.7
	X17682	Microtubule associated protein MAP2c	−2.1
	Z12152	Neurofilament protein middle (NF-M)	2
	AF031880	Light molecular-weight neurofilament (NF-L)	2.1
	X62952	Vimentin	3
Neurotransimission/ signal transduction	U67139	PSD-95/SAP90-associated protein-3	−8.3
	M36418	AMPA-selective glutamate receptor	−3.3
	AB020879	homer-3	−3
	L09119	C kinase substrate (RC3)	−2.6
	AA858621	CaM-kinase II inhibitor alpha	−2.4
	K03486	Protein kinase C type III	−2.2
	X06107	Insulin-like growth factor I	−8.2
	L01624	Serum and glucocorticoid-regulated kinase (sgk)	2.1
	AA945169	Transthyretin	5.5
	AI044508	Neuron specific gene family member 1	6.4
	V01244	Prolactin	9.1
Transcription regulation	AI180350	CTD-binding SR-like protein rA9	−10.9
	AI230572	nuclease sensitive element binding protein 1	−4.9
	D82074	neurogenic differentiation 1	3.3
Immunity/ oxidation	X13044	MHC-associated invariant chain gamma(cd74)	−16.3
	M15562	MHC class II RT1.u-D-alpha chain	−2.7
	S49491	Proenkephalin, opioid peptide	−4.8
	AF077354	Ischemia responsive 94 kDa protein	−7.7

Note: *Positive fold change values indicate an increase, and negative values indicate a decrease in gene expression of the memory-impaired rats compared to memory-unimpaired ones

Immunity and oxidative signaling　A number of immunity-related genes were down-regulated in both hippocampus and entorhinal cortex of memory-deficient rats. Major histocompatibility complex (MHC)-associated invariant chain gamma (cd74) and MHC class II RT1.u-D-alpha chain showed a reduced expression in the two brain regions of memory-impaired rats. In addition, several genes associated with oxidative response were differentially expressed in the hippocampus or entorhinal cortex of memory-deficient rats. Acyl-coA oxidase, the enzyme that catalyzes the oxidation of fatty acyl-CoA to trans-2-enoyl-CoA, was up-regulated in the hippocampus of memory-impaired rats. On the other hand, two oxidative stress response proteins, heat shock protein 27 and glutathione peroxidase, were down-regulated in the

hippocampus of memory-declined rats.

3 Discussion

In an effort toward the understanding of the molecular mechanism of age-related memory impairment, we systematically analyzed the gene expression in the hippocampus and entorhinal cortex in aged memory-impaired and memory-unimpaired rats. Our results revealed that genes involved in structure organization, neurotransmission, signal transduction, oxidative response and transcriptional regulation were regulated in age-dependent memory impairment. The expression level of microtubule associated protein MAP2c was significantly decreased in the memory-impaired rats. It is well known that MAP2c is a major component of cross-bridges between microtubules in dendrites and plays an important role in the stabilization of microtubules. Recently, it has been shown that MAP2 is an anchoring protein of PKA in dendrites. Loss of MAP2 function results in the reduction of dendritic PKA and decrease of CREB activity[25]. These results suggested that reduction of MAP2 might be one of the molecular events in the brain of memory-impaired rats, since both PKA and CREB play essential roles in learning and memory[13]. Vimentin, another structure protein, changed its expression in the hippocampus and entorhinal cortex of memory-impaired rats. Vimentin is a component of the intermediate filaments and a specific marker of astrocytes. Previously, we have found that vimentin was up-regulated in the hippocampus after fear memory[26]. Interestingly, vimentin was significantly down-regulated in the hippocampus of memory-impaired rats. NF-M and NF-L were up-regulated in entorhinal cortex. It has been reported that NF-M and NF-L were phosphorylated by cyclin-dependent protein kinase 5 (cdk5) and the phosphorylated neurofilament proteins were accumulated in Alzheimer's disease brain[27]. In addition, NF-L phosphorylation has been demonstrated to be associated with synaptic plasticity[28,29].

Our gene expression analysis showed that several genes involved in the neurotransmission, such as GABA receptor, AMPA receptor and serotonin receptor, changed their expression levels in the brain of aged memory-impaired rats. Although there is evidence suggesting that these receptors participated in learning and memory[30−32], our results further demonstrated that these genes could be associated with age-related memory impairment. Synaptic vesicle associated proteins regulate vesicle trafficking and neurotransmitter release. We have found that the expression of a number of these proteins were altered in memory-impaired animals, such as SVOP, Cplx2, and PSD-95-associated protein-3. It has been reported that Cplx2 is associated with the assembled SNARE and modulated synaptic vesicle exocytosis[33]. On the other hand, PSD-95-associated protein-3 interacts with PSD-95 protein to form a protein complex and plays an essential role in the neurotransmission[34]. We have found IGF signaling was down-regulated in the brain of memory-affected rats. IGF signaling pathway has been demonstrated to participate in many physiological processes, including cell proliferation and differentiation, aging and memory. Indeed, Igf-1 and 2 expressions in the brain are both decreased with aging[35,36]. Injection of IGF-I peptide into the brains of old rats can ameliorate age-related memory deficits[37]. Furthermore, IGF-I level is negatively associated with cognitive ability in human[38]. Since IGF signaling is involved in protein kinase activity, protein phosphorylation or dephosphorylation can be one of the mechanisms behind age-related memory impairment.

Indeed, a number of protein kinases and phosphatases, including cyclin-dependent kinase 5, protein kinase C type III, SGK, and protein phosphatase 2C, changed their expression levels in the brain of memory-impaired rats. Genes involved in another important signal pathway, calcium signaling, were also altered in memory-impaired rats. Homer 3, whose signal pathway mediates intracellular calcium release[39], and inositol-1, 4, 5-triphosphate receptor were down-regulated in the entorhinal cortex.

Much evidence indicated that long-term memory required new gene expression. It is therefore not surprising that a number of transcription factors changed their expression levels in hippocampus and entorhinal cortex of memory-deficient rats. It was found that zinc finger protein krox-20/egr-2, Msx-1 protein and Bromodomain adjacent toBAZ2B, were down-regulated. It is interesting to note that NF-YC, which forms a trimeric complex with NF-YB and NF-YA and binds to CCAAT motifs in promoter regions of a variety of genes and trans-activates their expression, was significantly up-regulated. Furthermore, it has been reported that NF-YC can bind to Smad2 and Smad3, two downstream transcription factors of TGF-beta signaling pathway, and suppress their activity[40]. In addition, we observed two other components of the TGF-beta/Smad signaling pathway, Dab2 and type III TGF-beta receptor, were down-regulated in hippocampus of memory-affected rats. These results suggested that TGF-beta/Smad signal pathway might play an important role in memory impairment. In summary, the gene expression profiling analysis revealed that many genes and signal pathways might participate in the age-related memory impairment. The results can serve as a starting point for more detailed functional studies of these genes in the brain.

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Supplementary Table　Primers and probes used in the real-time RT PCR experiments

Accession number	Gene name	Forward primer	Reverse primer	Taqman probe
AB016160	GABAB receptor 1c	CTGTTAAAAACCTGAAG CGTCAAG	CCGGGCTTCCGTC TCATAG	TGCTCGAATCATCG TGGGACTT
D83036	Msx-1 protein	AAGCAAGGAGCACAGCT CCTT	CCCCTCAGAGCAA TGCTTTG	CATTGTCAGACTCC AAACGCATTG
AI012051	BAZ2B	TGGAAAACTGCTATCCC CTACAC	TGTGGCGTCTCCC GAATC	CTGTTTCAAAGGCA GGCACTACCA
Z46882	TOAD-64	GGCCTAGATTTTGCAAC AGATTAGA	GCCAGAACAGACC GGAGACA	CTTTTGAAGGTTCT CTACCATTTTTC
V01244	Prolactin	TCAGCCCGGAAAGGTAT GTG	CCCATCGGTCATC AGGAATACT	CAGCTCATTGAATA AGTGGCTTTCT
AA894148	Apolipoprotein A-IV	GTCTGGGCTCCCCTTTT TTT	ACGGGCTTGGCGG AAT	CAGGGTCTTCTTTC CCCGCT
X62952	Vimentin	GCTGCAGGCCCAGATT CA	GGTGAGGTCAGGC TTGGAAA	AACAGCATGTCCAG ATCGATGTGG
AA945169	Transthyretin	GCTACTGCTTTGGCAA GATCCT	GTCGTCAGTAACC CCCAGAACT	CCTCCTGGGCTGGG TCCCT
AI102411	Brain-type clathrin light-chain	GCAGCCAGATGCTGAT ACCA	TCCTCCTTGGATT CTTTCACAAA	TGGCTATGTGGCAT CGGAAGAGGC
J04488	Prostaglandin Dsynthetase	TCAGGACTCCCGTGCT CTGT	GAGGGCGGAGAA GGTCTTTG	ACTCTTGAGACCCA AGCCCTGGC
L36664	Kininase II	CCCGGAAATACGAAGA ATTGC	TGGCTCTCCCCAC CTTGTC	TTGGGTGTGGAAG AGCTGGCA
AB017820	Klotho	CAACGGCTTCCTGGGT TCT	CGGTGCACACGGT GTACTCT	CGCTGGGAAGGTT TTGTCCG
AI171462	CD24	GCCTGGCCCGTGTCAA	ACACCCTCCTTCT GTGGCTTT	ACTAACAAAGGTCAA ATACAATATGTCAGA
J02752	Acyl-coA oxidase	CACGGGTCGTTGCTTT GG	ACGGAGGTCAGTG TTGGTGTT	TCACTTCTGTCGCCA CCTCCTC
AA892801	Elongation 2 factor	TCCTCAGGCTCCAGTT GCA	CCGTGCTGATGAT GAACAAGA	TTCCAGCAGGGCCCG GTCC