Microarray analysis of insulin-regulated gene expression in the liver: The use of transgenic mice co-expressing insulin-siRNA and human IDE as an animal model

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Abstract. To characterize the changes in global gene expression in the livers of H1/siRNA$_{insulin}$-CMV/hIDE transgenic (Tg) mice in response to the reduced bioavailability of insulin, total RNA extracted from the livers of 20-week-old Tg and non-Tg mice was converted to cDNA, labeled with biotin and hybridized to oligonucleotide microarrays. The microarray results were confirmed by a real-time reverse transcription-polymerase chain reaction. Two hundred and fifty-one and 73 genes were up- and down-regulated, respectively by insulin in H1/siRNA$_{insulin}$-CMV/hIDE Tg mice compared to the controls. Genes encoding for physiological processes, extracellular defense response and response to biotic stimuli were significantly over-represented in the up-regulated group. Among the down-regulated transcripts, those encoding for extracellular matrix proteins were dramatically over-represented, followed by those related to monooxygenase and oxidoreductase activities. The major genes in the up-regulated categories included Egr1, Saa2, Atf3, DNAJB1 and cCL2, whereas those in the down-regulated categories were Cyp17a1, Adn, Gadd45g, Eno3 and Moxd1. These results indicate that the microarray analysis identifies several gene functional groups and individual genes that respond to a sustained reduction in the insulin levels in the livers of Tg mice. These results also suggest that microarray testing is a useful tool for the better understanding of insulin-regulated diabetes-related diseases.

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Introduction

Insulin is a very crucial biomolecule, which rapidly decreases the blood glucose level and inhibits the production of endogenous glucose. This protein also exerts its potent effects on hepatic glucose fluxes via direct and indirect mechanisms (1-4). The direct effects can be further divided into 'acute' insulin actions, which lead to a rapid decrease in glucose production (5-7), and 'chronic' insulin actions, which regulate the gene expression of key gluconeogenic enzymes (8-10). Studies on dogs have indicated that the acute and direct effects of insulin on the liver mainly inhibit hepatic glycogenolysis (11). The indirect effects of insulin on the output of hepatic glucose include the suppression of lipolysis, inhibition of glucagon secretion and activation of hypothalamic descending pathways (12,13).

H1/siRNA$_{insulin}$-CMV/hIDE Tg mice (TG9385) had the chromosome integrated dual-recombinant expression system, containing insulin siRNA and human IDE under the control of the *H1 CMV* promoters, respectively (14-16). This system was first established in H1/siRNAEGFP-CAG/HcRed1 Tg mice co-expressing an enhanced red fluorescent protein and a suppressed green fluorescent protein (17). In the dual expression system, small interfering RNA or double-strand RNA (dsRNA)-based gene silencing is a highly conserved mechanism for sequence-specific post-transcriptional gene silencing between various species (18,19). This technique has been used in the nematode *Caenorhabditis elegans* to suppress gene expression by an injection of a double-strand mixture into adult animals (20). However, this method has a critical limitation in that the transfected synthetic siRNA works for only a few days in mammalian cells. This problem can be overcome by a vector-based technique for the synthesis of siRNA driven by the RNA polymerase III promoter (21-23). However, there are no reports as to whether the transgene-based siRNA system can suppress the expression of the insulin gene in adult mice.

In H1/siRNA$_{insulin}$-CMV/hIDE Tg mice, the highest insulin siRNA and hIDE expression levels were observed in the muscle, followed by the lung, brain, heart, liver, kidney and

intestine. Furthermore, the level and activity of hIDE mature proteins (110-kDa) were significantly higher in the liver, which is the primary target tissue for insulin clearance. These Tg mice also showed significant impairment of glucose tolerance during the course of an IPGTT. In particular, the 20-week-old Tg mice showed a greater impairment of glucose tolerance than their non-Tg counterparts, with a higher difference being observed in the male mice. The serum insulin level was significantly lower in the H1/siRNA$_{insulin}$-CMV/hIDE Tg than non-Tg mice (14,16).

In this study, we characterized the changes in global gene expression in the livers of H1/siRNA$_{insulin}$-CMV/hIDE Tg mice in response to reduced bioavailability of insulin.

Materials and methods

H1/siRNA$_{insulin}$-CMV/hIDE transgenic mice. The H1/ siRNA$_{insulin}$-CMV/hIDE Tg mice used in this study were developed in our laboratory by the microinjection of the male pronucleus of fertilized embryos, which were obtained by the crossing of C57BL/6 (female) mice with DBA2 (male) mice during 2005. The injected egg was then transferred into the oviducts of a pseudopregnant ICR recipient female. The transgene was identified by DNA-PCR analysis of the genomic DNA isolated from the tails of the 4-week-old founder mice. The H1/siRNA$_{insulin}$-CMV/hIDE DNA was synthesized using the sense and antisense primers, 5'-GGT TTT CCC AGT CAC GAC-3 and 5'-GAG TTA GCT CAC YCA TAG GC-3, respectively, with a complementary H1/ siRNA$_{insulin}$-CMV/hIDE vector, ranging from 359-376 and 663-683 nucleotides, as the DNA template. After 25 cycles of amplification, the level of the PCR product (324-bp) was quantified on 1% agarose gels using a Kodak electrophoresis documentation and analysis system 120. A total of ten 22-week-old mice, five Tg and five non-Tg were used in this study.

All the mice were kept in an accredited Korea FDA animal facility in accordance with the AAALAC International Animal Care policies (Accredited Unit-Korea Food and Drug Administration, unit no. 000996). The mice were given a standard irradiated chow diet (Purina Mills Inc.) *ad libitum*, and maintained in a specified pathogen-free state under a strict light cycle (light on at 06:00 h and off at 18:00 h). All pedigrees were hemizygous for their transgene.

RNA isolation. The livers from 22-week-old Tg mice and their non-Tg littermates were used for isolation of total RNA using RNAzol (Tel-Test Inc., CS104) according to the manufacturer's instructions. The frozen tissues were chopped with scissors and homogenized in RNAzol B solution using a glass-Teflon homogenizer. Chloroform, 0.2 vol, was added to the mixture, shaken vigorously for 15 sec and then incubated on ice for 5 min. After incubation on ice, the homogenate was separated into two phases by centrifugation at 12,000 rpm for 15 min. The aqueous phase was transferred to a fresh tube and precipitated with an equal volume of isopropanol. The RNA pellet was suspended in DEPC (diethylpyro-carbonate)-treated dH$_2$O and purified using a QIAquick purification kit (Qiagen, CA). The integrity of the 18S/28S rRNA was analyzed using a Bioanalyzer 2100 (Agilent), with the RNA quality checked by the ratio of the absorbance at 260 nm to that at 280 nm using a biophotometer (Eppendorf, Germany).

cRNA synthesis and labeling. cRNA synthesis and labeling were performed using a Chemiluminescent RT-IVT labeling kit (Applied Biosystems, CA) according to the manufacturer's instructions. Six individual samples were submitted in randomly assigned pairs representing tissues from non-Tg and Tg mice. Three micrograms of total RNA was used to synthesize cDNA. Total RNA was incubated with T7-oligo(dT) primer in nuclease-free water for 10 min at 70˚C. The resulting solution was continuously incubated for 2 h at 42˚C in a reaction mixture containing RNase inhibitor, 10X 1st strand buffer mix and reverse transcriptase. Double-strand cDNA was synthesized for 2 h at 16˚C in a reaction mixture containing nuclease-free water, 5X 2nd strand buffer and 2nd strand enzyme, and then purified using a purification column. Finally, the biotinylated cRNA was generated from the cDNA using a BioArray cRNA high yield RNA transcript kit containing purified double-strand cDNA, 5X IVT buffer mix, DIG-UTP and IVT enzyme mix.

Oligonucleotide array expression analysis. Insulin-regulated gene expression analysis in the liver was conducted using the Mouse Genome Survey microarray (Applied Biosystems, CA) containing oligonucleotide probes for 33,315 genes. The cRNA was fragmented in 5X fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM KOAc and 150 mM MgOAc) for 30 min at 60˚C prior to chip hybridization. Fifteen micrograms of fragmented cRNA was then added to the hybridization cocktail (0.05 μg/μl fragmented cRNA, 50 pM control oligonucleotide, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 100 mM MES, 1 M Na$^+$, 20 mM EDTA and 0.01% Tween-20). Each sample was hybridized to a separate oligonucleotide array for 16 h at 55˚C in the GeneChip hybridization oven 640. After hybridization, the solution was removed and the slides washed twice with 2X SSC containing 0.1% SDS for 5 min at 42˚C. The slides were incubated for 20 min with the anti-Dig-AP in CL blocking buffer, and then developed using the chemi-luminescence substrate. Thereafter, the hybridized arrays were scanned using an AB 1700 analyzer. Scanning and basic analysis were performed using the GenPlex software release 1.0 (Istech Inc., Korea).

Real-time PCR analysis of representative genes. The expression of the representative genes was examined using real-time PCR analysis with 5 μg of the total RNA from each tissue. For reverse transcription, 500 ng of the oligo-dT primer [Gibco BRL (18418-012)] was annealed for 10 min at 70˚C. The complementary DNA, which served as the template for further amplification, was synthesized by the addition of dATP, dCTP, dGTP, dTTP and 200 units of reverse transcriptase. After reverse transcription, real-time PCR analysis was carried out in triplicate for each mixture, with the addition of the synthesized cDNA to TaqMan Universal master mix and CYP AdB product (mixture of sense, antisense, and FAM primer). PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an endogenous
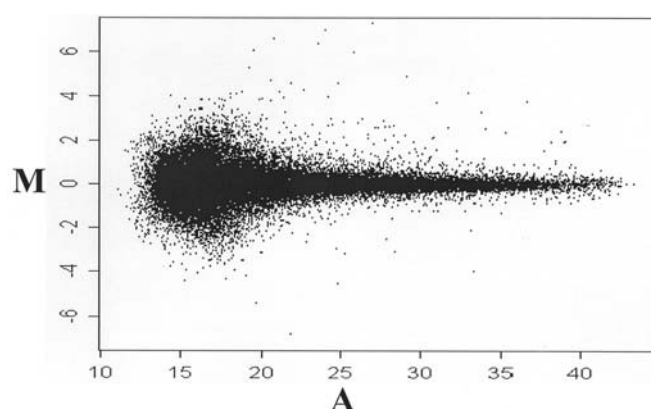
Figure 1. MA plots of cDNA microarray hybridization. Gene expression signals of H1/siRNA$_{insulin}$-CMV/hIDE Tg and non-Tg mice. Each point represents the average expression value for the same gene from the non-Tg and Tg mice. MA plots were used to represent the R and G data, where M=log2R/G and A=log2 (RxG). M, expression ratio; A, the false color image; G, the stronger control sample hybridization (Cy3); and R, the stronger transgenic sample hybridization (Cy5).

control, was performed simultaneously, with the exception of the rodent GAPDH control reagent (AB4308313). TaqMan probes have two fluorescent dyes, one at the 5'-terminal (Reporter, R) to displace the strand, and the other at the 3'-terminal (Quencher, Q) to block the extension. The reaction mixture was then subjected to amplification in the following sequence: 1 cycle at 50˚C for 2 min (annealing stage), 1 cycle at 95˚C for 10 min (denaturation stage), 40 cycles at 95˚C for 15 sec and at 60˚C for 2 min (extension stage). In all cases, an initial denaturation was carried out at 95˚C for 5 min to monitor the decrease in the formation of a primer-dimer complex under these PCR conditions. The ABI-SDS was programmed to take R and Q fluorescent dye readings after each cycle at a temperature several degrees (60˚C) lower than the melting temperature of the target amplicon; this step was undertaken to avoid, or at least minimize, any potential contribution to the overall fluorescent dye signals due to primer-dimer formation. A calibration curve was constructed by plotting the R/Q ratio against the amounts of each cDNA synthesized, based on the RNA isolated from the livers of both the Tg and non-Tg mice, with the latter used as the control.

Statistical analysis. Testing for significance was performed using a one-way ANOVA test of variance (SPSS for Windows, release 10.10, standard version, Chicago, IL). All values were represented as the mean ± standard deviation (SD), with the mean difference between dietary treatments evaluated at $P<0.05$.

Results

Image of the gene expression profile on cDNA microarray. A microarray analysis plot summary of the microarray gene expression data represented on the Mouse Genome Survey microarray (33,315) is shown in Fig. 1. The location of average gene expression values along the first diagonal showed a non-systematic bias between the non-Tg and Tg groups. A higher variance was observed at lower than higher expression levels. A fairly considerable number of genes were also expressed at elevated or reduced levels in the Tg compared to the non-Tg livers (Fig. 1), although the expression levels of most genes were different between the non-Tg and Tg livers. Based on the arbitrary difference in the 2-fold change, 152 genes were revealed to be up-regulated and 73 genes down-regulated in the Tg compared to the non-Tg livers.

Ontology categories of insulin-regulated gene expression. To analyze the ontology classification of the insulin-regulated gene expression in the livers of Tg mice, gene lists were categorized into functional clustering according to the Gene ontology classification using the OntoExpress on-line software; the results are presented in Tables I and II. OntoExpress identified 9 GO categories showing significantly elevated expression in the list of up-regulated transcripts (Table I). As presented in Table I, the largest number of transcripts turned out to be the proteins mainly associated with physiological processes, the extracellular defense response and biotic stimulus. From the list of down-regulated transcripts, OntoExpress identified 5 markedly overexpressed GO categories (Table II). In this group, the transcripts of those genes located on extracellular matrix were especially highly expressed, followed by those involved in mono-oxygenase and oxidoreductase activities. These results suggest that insulin can regulate the expression of genes

Table I. Gene ontology categories significantly (p<0.05) over-represented among the 152 gene transcripts up-regulated in the livers of H1/siRNA$_{insulin}$-CMV/hIDE Tg mice.

P-value	Number of transcripts	Percent	GO category
0.0004	99	39.44	Physiological processes
0.0363	35	13.94	Extracellular
0.0006	15	5.97	Defense response
0.0017	15	5.97	Response to biotic stimulus
0.0018	12	4.78	Immune response
0.0014	9	3.58	Response to pest/pathogen/parasite
0.0402	5	1.99	Lipid binding
0.0446	3	1.19	Lipid transporter activity
0.0327	3	1.19	Protein tyrosine/serine/threonine phosphatase

Table II. Gene ontology categories significantly (p<0.05) over-represented among the 73 gene transcripts down-regulated in the livers of H1/siRNA$_{insulin}$-CMV/hIDE Tg mice.

P-value	Number of transcripts	Percent	GO category
0.0405	5	6.84	Extracellular matrix
0.0209	4	5.48	Monooxygenase activity
0.0494	3	4.11	Oxidoreductase activity
0.0395	2	2.74	Oxidoreductase activity miscellaneous
0.0408	2	2.74	Non-G-protein-coupled 7TM receptor

Table III. Genes with the highest fold increase among those (152) up-regulated in the livers of H1/siRNA$_{insulin}$-CMV/hIDE Tg mice.

Name	GeneBank ID	Fold increase	Description
Egr1	M19643	9.66	Nucleic acid binding; transcription factor activity
Saa2	U60438	8.20	Lipid transporter activity; protein binding
Atf3	BC019946	7.84	DNA binding; transcriptional repressor activity
Saa2	U60438	7.32	Lipid transporter activity; protein binding
Dnajb-l	AB028272	6.54	Heat shock protein binding; unfolded protein binding
Tff3	BC011042	5.90	-
Copeb	AY027436	5.66	Nucleic acid binding; zinc ion binding
Gadd45a	L28177	5.66	Protein binding
Ccl2	M19681	5.48	G-protein-coupled receptor binding; cytokine activity
Cxcl-IO	AF227743	5.42	Cytokine activity; chemokine activity
Nr4a1	J04113	5.36	Transcription factor activity; receptor activity
Lamb3	AK078970	5.28	Structural molecule activity; protein binding
Slpi	AF002719	5.26	Endopeptidase inhibitor activity
ler5	AF079527	5.20	-
Maff	BC022952	5.18	DNA binding
Orm2	M17376	5.12	Transporter activity; binding
Trib1	AF358866	5.12	Nucleotide binding; protein kinase activity
Sh2d2a	AK037818	4.92	SH3/SH2 adaptor activity
Zfp112	AF167319	4.76	Nucleic acid binding
Hoxa5	M36604	4.64	DNA binding; transcription factor activity
Mt1	AK011040	4.62	Copper ion binding; zinc ion binding; metal ion binding
ler3	AK051003	4.50	-
Myc	L00039	4.48	DNA binding; transcription factor activity
Cyp2b13; Cyp2b9	M21855	4.48	Monooxygenase activity; iron ion binding
Rasd-l	AF009246	4.24	Nucleotide binding; GTP binding
Dusp6	AK088468	4.22	Phosphoprotein phosphatase activity
LOC277830	BC013476	4.12	Monooxygenase activity; oxidoreductase activity
Hmoxl	X13356	4.10	Heme oxygenase (decyclizing) activity
S100a9	M83219	4.08	Calcium ion binding

related to physiological processes, the xenobiotic defense response, extracellular matrix and monooxygenase activity in the livers of Tg mice.

Genes with the highest expression levels of up-regulation and down-regulation. In order to characterize the genes showing the highest overexpression, those with at least a 4-fold increase were selected from the 152 up-regulated and 75 down-regulated genes. As shown in Table III, the highest increase in transcripts showed Egr1 (9.66-fold) to be responsible for the transcription factor activity, followed by Saa2, Atf3, Dnajb1 and Tff3. The various transcriptional factors and transporters were especially significantly elevated by the regulation of insulin in the livers of Tg mice. Also, 20 genes with the highest fold decreases were significantly overexpressed (Table IV). Of these genes, transcripts of cytochrome family coding genes (Cyp17a1 and Cyp2c) were most dramatically decreased, followed by those associated with proteolysis, regulation of the cell cycle, glycolysis and oxidoreductase activity. These results indicate that insulin

Table IV. Genes with the highest fold increase among those (73) down-regulated in the livers of H1/siRNA$_{insulin}$-CMV/hIDE Tg mice.

Name	GeneBank ID	Fold increase	Description
Cyp17a1	M64863	5.96	Electron transport; hormone biosynthesis
Adn	M13386	5.28	Proteolysis; immune response
Gadd45g	AF055638	5.04	Regulation of progression through cell cycle
Eno3	M20745	4.52	Glycolysis
Cyp2c70	BC022151	4.20	Oxidoreductase activity
Stomll	AK045820	3.82	-
Moxd1	AB041606	3.74	Catecholamine metabolism
Sertad3	AF317202	3.74	Transcription
Cck	M11739	3.72	Neuron migration; axonogenesis
Vrk2	AF513620	3.64	Protein amino acid phosphorylation
Cyp26a1	AF115769	3.48	Electron transport; retinoic acid metabolism
Sntb2	AK010705	3.40	-
Olfr872	AY318050	3.22	Signal transduction
Npl	AK088859	3.18	Biological process unknown
Fzd8	U43321	3.16	Signal transduction
Btbd14a	AK043047	3.16	-
Pdk3	AK075879	3.16	Protein amino acid phosphorylation
Foxa2	X74937	3.10	Branching morphogenesis; transcription
Drd-lip	AK003400	3.02	Dopamine receptor signaling pathway
Fzd8	U43321	3.00	Signal transduction

Table V. Comparison of the expression ratios of selected genes from the livers of H1/siRNA$_{insulin}$-CMV/hIDE Tg and non-Tg mice, as determined by real-time PCR and microarray analysis.

Name	GeneBank ID	TG/WT ratio	
		Real-time PCR	Microarray
Apolipoprotein A	M64249	3.66	3.62
Defensin-related cryptdin 4	AF178040	4.31	2.46
Fatty acid binding protein 4	BC018558	1.60	2.06
Cytochrome P450 family 17	M64863	-4.11	-5.96
Stearoyl-coenzyme A desaturase 1	BC007474	-3.73	-2.62
Nephronectin	BC068308	-2.22	-2.18
Cytochrome P450 family 2	BC022151	-3.12	-4.10

can be closely involved in the regulation of genes for transcription factors, transporters, cytochrome and glycolysis under the condition of a low insulin level.

Comparison of expression ratios of selected genes. In an attempt to compare the expression ratios of several genes from the liver, the expression levels of selected genes were examined by real-time PCR. Seven of the up- and down-regulated genes judged from GeneChip data were selected, and also analyzed by real-time PCR. Table V shows the fold changes in each gene expression in the H1/siRNA$_{insulin}$-CMV/hIDE Tg mice compared to those in the control mice when analyzed by the GeneChip and real-time PCR. The changes in these levels of gene expression were similar in

direction and magnitude between the two techniques. These results suggested that the expression ratios from the microarray in selected genes were very similar to those from real-time PCR.

Discussion

IDE (insulysin, insulinase, EC3.4.24.56) is a member of the M16 family of zinc-metalloendopeptidase, which contains a tetrad of conserved residues (H-x-x-E-H-x (76)-E) at its catalytic center (24). This protein is broadly expressed in both insulin-sensitive and insulin-insensitive tissues, and localized mainly in the cytoplasm of cells. However, different forms of this protein can be secreted into the extracellular

space or associated cell surface (25,26). Until recently, several studies reported various IDE high-affinity substrates, which were polypeptides, 28-51 amino acids in length. A variety of physiological peptides have been shown, *in vitro*, to be substrates for IDE, including insulin and glucagons (27), the atrial natriuretic factor (28), transforming growth factor α (29,30), atrial natriuretic peptide (31), ß-endorphin and dynorphins (32), amylin (33), and amyloid ß peptides (34). IDE may also have other biological functions, such as the clearance of the ß-amyloid precursor protein in the intracellular domain (AICD), which is suggested to regulate the transcriptional activity in the nucleus (35). Furthermore, IDE has been demonstrated to directly interact and enhance the DNA binding of androgen and glucocorticoid receptor fragments *in vitro* (36). In a recent report, Duckworth *et al* (37) reported that IDE exists as a complex with the 26S proteasome in cells, which is regarded as the principal site of the ubiquitin-dependent intracellular protein. In this study, we characterized the changes in the global gene expression in the livers of H1/siRNA$_{insulin}$-CMV/hIDE Tg mice in response to the reduced bioavailability of insulin. Therefore, the results obtained from the microarray analysis were also due to the effect of overexpressed IDE protein.

Microarray techniques are widely used analytical methods for the detection of changes in the global gene expression in various disease animal models. In particular, this technique has previously been applied to the study of androgen-regulated gene expression in the testis of androgen-binding protein Tg mice. Three hundred and eighty-one genes were up-regulated and 198 down-regulated in the androgen-deficient animals compared to the control (38). In this study, we analyzed the global gene expression in the livers of H1/siRNA$_{insulin}$-CMV/hIDE Tg mice, which showed a lower circulating insulin level. The Tg mice used in this study showed quite different diabetic symptoms compared with other diabetic animal models. Until now, most animal models have shown inhibition of the synthesis and signal pathway of insulin due to disruption of its receptor. However, the insulin level in these Tg mice was significantly decreased, but did not induce damage or disruption to pancreatic tissue. Furthermore, the insulin secreted in the pancreas was rapidly degraded by overexpressed hIDE in the liver (16,17).

Recently, Colombo *et al* (39) investigated changes in the gene expression in skeletal muscle, liver, fat and pancreatic islets after energy restriction in male Zucker diabetic fatty rats. In our study, the expression of 123 genes was increased, while that of 103 was decreased, in liver tissue related to lipid metabolism. Wilson *et al* (40) also suggested that 46 genes with lower expression and 8 with higher expression were identified in the kidneys of NOD diabetic mice compared to the NOD nondiabetic control. These genes belong to diverse functional groups, including oxidative phosphorylation, free radical neutralization, channels, pumps, lipid processing, translation and transcription. Furthermore, the gene expression profile was evaluated in the skeletal muscle of type 2 diabetic patients. The transcriptional patterns of 85 genes were shown to be altered in the diabetic patients after withdrawal of insulin treatment compared with those in the nondiabetic control subjects. These altered transcripts were involved in insulin signaling, transcription factors and mitochondrial

maintenance (41). In our study, 152 genes were identified as up-regulated and 73 as down-regulated in the livers of H1/siRNA$_{insulin}$-CMV/hIDE Tg versus non-Tg mice. A total of 29 up-regulated genes appeared to have 4-fold increases in their expression levels. Of the up-regulated transcripts, as shown in Table III, Egr1, responsible for the transcriptional activity by nucleic acid binding, was most significantly over-represented (9.66-fold), followed by Saa2, Atf3, Dnajb1 and Tff3. Especially, various transcriptional factors and transporters were significantly increased by insulin regulation in the livers of Tg mice. Conversely, of the 20 transcripts with the greatest down-regulation (Table IV), those of the cytochrome family coding genes (Cyp17a1 and Cyp2c) were decreased most dramatically, followed by those related to proteolysis, regulation of cell cycle, glycolysis and oxido-reductase activity.

It is very important to confirm the expression ratio of genes from the microarray to fundamentally validate experimental data, which can be performed by quantitative RT-PCR and real-time PCR analysis of selected genes (38,42). In this study, we chose seven of the up- and down-regulated genes as judged from the GeneChip data, which were also analyzed by real-time PCR. The fold changes in each gene expression were similar in direction and magnitude between the two techniques (Table V).

Collectively, these results suggest that microarray analysis can identify several functional groups of genes as well as individual genes that respond to sustained reduction of insulin levels in the Tg liver. Also, these results suggest specific avenues for research toward a better understanding of how insulin regulates diabetes-related diseases.

Acknowledgements

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