

# Analysis of gene expression profiles in insulin-sensitive tissues from pre-diabetic and diabetic Zucker diabetic fatty rats

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## Abstract

Insulin resistance occurs early in the disease process, preceding the development of type 2 diabetes. Therefore, the identification of molecules that contribute to insulin resistance and leading up to type 2 diabetes is important to elucidate the molecular pathogenesis of the disease. To this end, we characterized gene expression profiles from insulin-sensitive tissues, including adipose tissue, skeletal muscle, and liver tissue of Zucker diabetic fatty (ZDF) rats, a well characterized type 2 diabetes animal model. Gene expression profiles from ZDF rats at 6 weeks (pre-diabetes), 12 weeks (diabetes), and 20 weeks (late-stage diabetes) were compared with age- and sex-matched Zucker lean control (ZLC) rats using 5000 cDNA chips. Differentially regulated genes demonstrating > 1.3-fold change at age were identified and categorized through hierarchical clustering analysis. Our results showed that while expression of lipolytic genes was elevated in adipose tissue of diabetic ZDF rats at 12 weeks of age, expression of lipogenic genes was decreased in liver but increased in skeletal muscle of 12 week old diabetic ZDF rats.

These results suggest that impairment of hepatic lipogenesis accompanied with the reduced lipogenesis of adipose tissue may contribute to development of diabetes in ZDF rats by increasing lipogenesis in skeletal muscle. Moreover, expression of antioxidant defense genes was decreased in the liver of 12-week old diabetic ZDF rats as well as in the adipose tissue of ZDF rats both at 6 and 12 weeks of age. Cytochrome P450 (CYP) genes were also significantly reduced in 12 week old diabetic liver of ZDF rats. Genes involved in glucose utilization were downregulated in skeletal muscle of diabetic ZDF rats, and the hepatic gluconeogenic gene was upregulated in diabetic ZDF rats. Genes commonly expressed in all three tissue types were also observed. These profilings might provide better fundamental understanding of insulin resistance and development of type 2 diabetes.

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## Introduction

Type 2 diabetes mellitus is a common metabolic disease involving abnormal carbohydrate regulation and lipid metabolism by insulin (Saltiel 2001). Insulin resistance, characterized as decreased insulin action on glucose uptake and metabolism, occurs prior to the onset of type 2 diabetes and plays a major role in its development. Contributing to insulin resistance, obesity is known as a strong risk factor for type 2 diabetes. However, obesity alone does not account for disease development, as only a small fraction of obese people develop diabetes, with most being able to maintain a euglycemic state despite also being insulin resistant. Since it is likely that the development of type 2 diabetes is determined in part by genetic factors, understanding differences in gene expression of insulin-sensitive tissues from animals in different progressive stages of diabetes development (e.g.

insulin resistant pre-diabetes and diabetes) may help uncover the underlying molecular mechanisms involved in disease progression.

A recent advancement in genomic research, microarray technology allows for the expression of thousands of genes to be quickly and easily monitored in parallel. Global gene expression, or transcriptional profiling, has been used to identify molecular markers for various pathological states and can be used to generate novel hypotheses to characterize different disease states. Studies using microarrays have been performed to identify candidate genes related to insulin resistance and diabetes in animal and human models of diabetes (Yang *et al.* 2002, Sreekumar *et al.* 2002, Lan *et al.* 2003, Rome *et al.* 2003, Mootha *et al.* 2003). However, until recently, there was no full set of alterations in gene expression that may possibly be involved in the disease pathology of type 2 diabetes.

A well-characterized model of obesity and type 2 diabetes, the Zucker diabetic fatty (ZDF) rat, has a point mutation in the leptin receptor that leads to impairment of the signaling capabilities of this receptor. The ZDF rat develops an age-dependent diabetes phenotype, with onset of obesity at 5–7 weeks of age accompanied by a metabolic state of early diabetes mellitus with hyperinsulinemia and insulin resistance, but with euglycemia. The full syndrome of diabetes develops at 10–12 weeks with hyperglycemia. The diabetic pathogenetic features manifested in this animal model are in many ways reminiscent of the pathogenesis of type 2 diabetes in humans. Therefore, the ZDF animal model is ideal for identifying molecules contributing to insulin resistance related to obesity and to elucidate the molecular pathogenesis of type 2 diabetes.

In this study, using cDNA microarrays, global gene expression in adipose tissue, skeletal muscle, and liver tissue were profiled from 6-week old pre-diabetic ZDF rats, 12-week old diabetic ZDF rats and 20-week old late-stage diabetic ZDF rats compared with age and sex-matched Zucker lean control (ZLC) rats. This approach provided several candidate genes and possible molecular mechanisms responsible for insulin resistance and progression to type 2 diabetes.

## Materials and methods

### Animals

Male Zucker diabetic fatty (ZDF/Gmi-*fa/fa*) rats (6, 12 or 20 weeks old) and their sex- and age-matched Zucker lean control (ZLC/Gmi-*+/fa*) rats were purchased from Genetic Models (GMI, Indianapolis, IA, USA). Animals were maintained on a commercial chow diet *ad libitum* before being killed. Body weights and blood glucose levels for the 6- and 12-week old ZDF rats were pre-diabetic and diabetic stage-appropriate, respectively. The rats were sacrificed by cervical dislocation, and the epididymal fat, skeletal muscle of the femoral region, and liver were removed and subjected to total RNA extraction.

### RNA preparation and labeling

Total RNA was isolated using the TRIzol reagent (GibcoBRL, Invitrogen Corporation, Carlsbad, CA, USA). The cDNA were labeled with the 3DNA Array kit (Genisphere, Hatfield, PA, USA) according to the manufacturer's protocol and recommendations. The cDNA of ZDF rats was labeled with Cy5, and cDNA of each paired control ZLC rat was labeled with Cy3. Briefly, total RNA (20 µg) and the Cy3 or Cy5 capture sequence primer were solubilized in 20 µl of diethyl pyrocarbonate (DEPC)-treated water and incubated for

10 min at 80 °C. The RNA mixture was then transferred on to ice and the RNase inhibitor was added. An equal volume of reaction mixture containing 5X first-strand buffer, 100 mM DTT, 10 mM dNTP mix and 200 units of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was added and the reaction was incubated for 2 h at 42 °C. The reaction was terminated by adding 0.5 M NaOH/50 mM EDTA and incubated at 65 °C for 10 min. Finally, the reaction was neutralized with 1 M Tris-HCl, pH 7.5. The cDNA samples were concentrated by centrifugation using Centricon filters (Millipore, Billerica, MA, USA).

### cDNA microarray analysis

The cDNA microarray analysis was performed on rat expression glass microarrays (Genomic Tree, Taejon, Korea), which were spotted with 5000 cDNA of known rat genes and expressed sequence tags (ESTs). A total of 36 microarray slides (3 ages × 3 tissue types × 4 replicates) were used to monitor gene expression levels. Hybridization and washing of the microarrays were carried out according to the manufacturer's instructions. In brief, cDNA probe solutions containing 2X hybridization buffer and Array 50 dT blocker were applied to the microarrays, which were then covered with a spaced glass coverslip and placed in a humidified chamber at 65 °C for 16 h. Then, the microarrays were sequentially washed for 15 min each, once at 65 °C in 2 × SSC and 0.2% SDS, once in 2 × SSC at room temperature, and once in 0.2 × SSC at room temperature, and then washed for 2 min at room temperature in 95% ethanol to fix the cDNA molecules to the probes. Then, the microarrays were centrifuged for 2 min at 1000 r.p.m. to dry the slide. In preparation for 3DNA hybridization, the 3DNA Capture Reagent and the anti-fade reagent were added to the hybridization buffer. The prepared hybridization buffer was added to the arrays and incubated for 2 h in a dark humidified chamber at 65 °C. The microarrays were again sequentially washed and dried.

### Scanning and data analysis

The hybridization images were quantitated by GenePix Pro 4.0 (Axon Instruments, Union City, CA, USA). The average fluorescence intensity for each spot was calculated and local background was subtracted. All data normalization and statistical analysis were performed using GeneSpring 6.1 (Silicon Genetics, Redwood, CA, USA). Genes were filtered according to their intensity in the control channel based on the two-component model for estimating variation from control strength (Rocke & Durbin 2001). Intensity-dependent normalization (LOWESS) was performed, where the ratio was

reduced to the residual of the LOWESS fit of the intensity vs ratio curve. In addition, to adjust the data from three different ages, we performed median centering by dividing each gene's signal ratio by the median of all the signal ratios for that particular gene. The values of fold change were calculated by dividing the median of normalized signal channel intensity (Cy5) by the median of normalized control channel intensity (Cy3). The ANOVA test (parametric) was performed at  $P$  values  $< 0.05$  to find genes that differentially expressed across conditions. Unsupervised hierarchical cluster analysis was performed by similarity measurements based on Pearson correlations around zero.

### Real-time reverse transcription PCR (RT-PCR)

For real-time RT-PCR, cDNA was prepared from 3  $\mu\text{g}$  of total RNA from ZDF or ZLC rats using random hexamers and the Moloney murine leukemia viral reverse transcriptase (Promega, Madison, WI, USA) in a final reaction volume of 20  $\mu\text{l}$ . The resulting cDNA was analyzed by real-time PCR using SYBR Green PCR core reagents (PE Biosystems, Warrington, UK). PCR was performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) and each gene-specific primer set was used at concentrations of 150 nM in a final volume of 25  $\mu\text{l}$ . The accuracy of the PCR products was verified by agarose gel electrophoresis. Quantification of a given gene, expressed as relative mRNA level compared with control levels, was calculated after normalization to 18S rRNA and using the  $\Delta\Delta C_T$  formula as described by Perkin Elmer. Individual  $C_T$  values were calculated as means of duplicate or triplicate measurements.

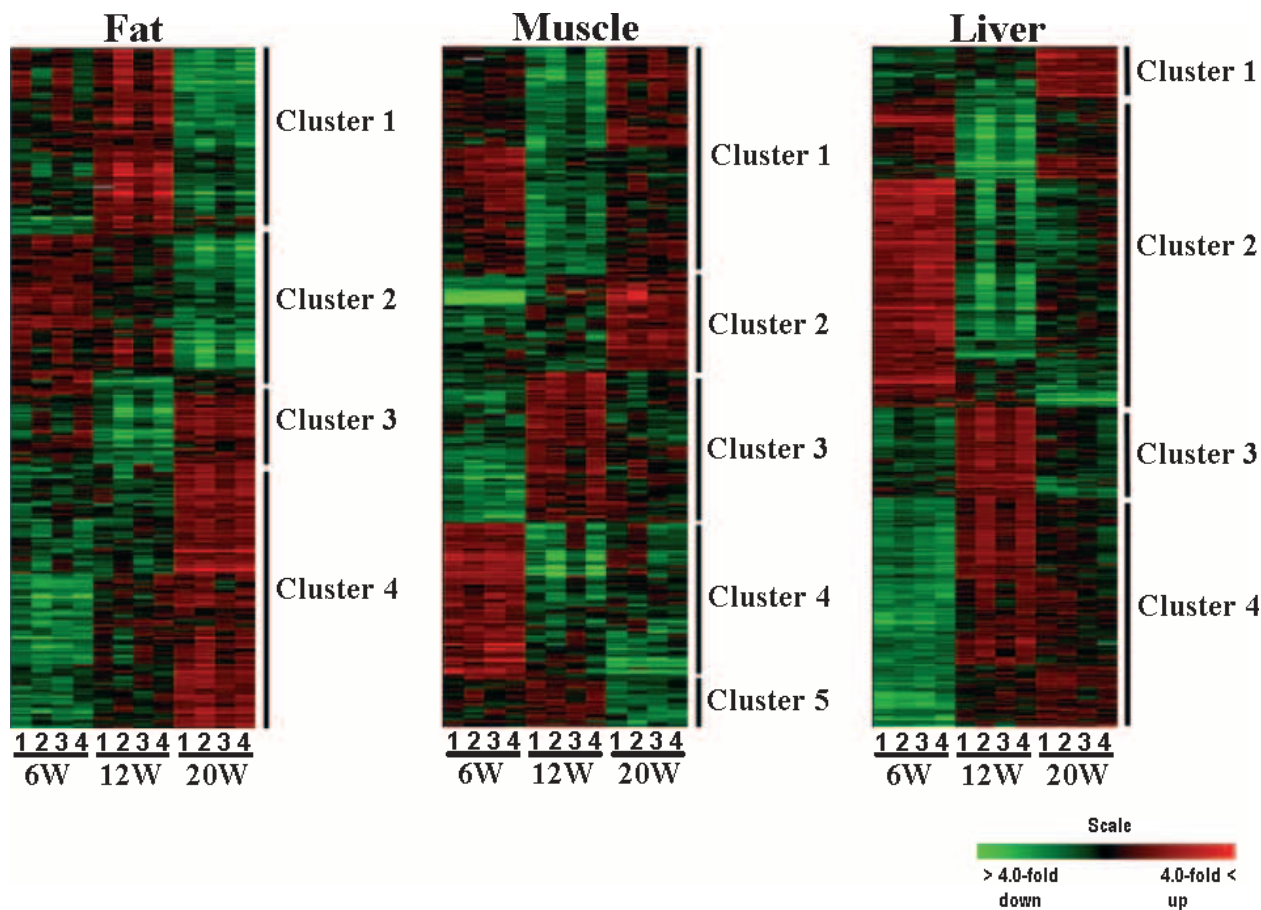
### Western blotting

Skeletal muscle or liver tissues from ZDF (6 and 12 weeks) and ZLC (6 and 12 weeks) rats were solubilized in RIPA buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu\text{g}/\text{ml}$  aprotinin, 1  $\mu\text{g}/\text{ml}$  leupeptin, and 1  $\mu\text{g}/\text{ml}$  pepstatin) for 30 min on ice. The lysates were clarified by centrifugation and separated by SDS-PAGE (10%), transferred onto nitrocellulose filter. The filters were probed with SREBP-1 (H-160) or PPAR $\gamma$  (H-100) antibody purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) for 16 h. Membranes were washed and incubated with a 1:4000 dilution of horseradish peroxidase conjugated secondary antibodies. Protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

## Results

### Overview of gene expression profiles in ZDF rats

Six-week old ZDF male rats, together with 6-week old ZLC rats serving as controls, were used to study pre-diabetic changes, whereas 12-week old ZDF male rats and age-matched ZLC rats were used to study diabetic changes, and 20-week old ZDF male rats were used to study late-stage diabetic changes. Microarrays were carried out to study gene expression in adipose tissue, skeletal muscle, and liver tissue from ZDF and ZLC rats using cDNA chips containing 5000 rat genes. In this study, four ZDF and ZLC rats at each age were used. Total RNA was isolated from three different tissues from ZDF or ZLC rats of corresponding ages. Four pairs of each Cy5-labeled ZDF and Cy3-labeled ZLC rat cDNA samples were applied to a cDNA chip respectively, which was essentially equivalent as applied in four replications. After performing intensity-dependent normalization on spots of 12 slides of each tissue (3 ages  $\times$  4 replicates), fold-changes were determined by dividing the median normalized signal channel intensity by the median normalized control channel intensity. Differentially expressed genes ( $P < 0.05$ ) as determined by ANOVA and with about a 1.3 fold-change in expression levels were selected. Differences observed at 6 weeks, which corresponds to the period preceding diabetes onset, were considered to be involved in causing type 2 diabetes, whereas those seen at 12 weeks were considered likely to be involved in the progression of type 2 diabetes. There were 381 genes found in adipose tissue, 319 genes from skeletal muscle, and 528 genes from liver that exhibits significant changes of expression during type 2 diabetes. These genes were further analyzed using hierarchical clustering to group genes with similar changes in expression profiles. As shown in Fig. 1, hierarchical clustering revealed that four replicates showed little variance of expression in each age for the three tissues, indicative of high reliability of chip data. Furthermore, the differentially expressed genes in adipose, skeletal muscle and liver were clustered into 4, 4, and 5 groups, respectively. Many of the differentially expressed genes in adipose tissue had a similar expression pattern in 6-week old pre-diabetic ZDF rats and 12-week old diabetic ZDF rats, whereas many genes in the liver and skeletal muscle had reverse expression patterns at 6 and 12 weeks of ages. Namely, many of the genes that were up- or downregulated in the liver and skeletal muscle of pre-diabetic ZDF rats became down- or upregulated reversely in diabetic 12-week old ZDF rats. Therefore, genes whose expression was differentially regulated from 6 to 12 weeks may contribute to the development of type 2 diabetes, along with up- or downregulated genes found in the tissues of diabetes-stage ZDF rats. Tables 1 to 3 list the representative genes in each cluster along with the putative biological



**Figure 1** Hierarchical Clustering Analysis of differentially expressed genes in insulin sensitive tissues. All replicated ratios of gene expression levels (shown with the Arabic numerals 1 to 4) are displayed colorimetrically in the three ages (6-week, 6W; 12-week, 12W; 20-week, 20W) old of ZDF rats compared with the control. The scale bar at the bottom represents the relative level of expression of each gene. (Red) Increase and (green) decrease in expression level relative to the median value at specific ages of animals. Details of gene identity and expression differences are given in the Tables 1 to 3.

functions of their encoded proteins, as found in searches of the public database. Through quantitative real-time RT-PCR, we confirmed expression levels of genes selected on the basis of biological interest or ranking *P* value in each tissue from cross counterpart animals. As shown in Fig. 2, the expression of genes selected in skeletal muscle and liver was concordant with microarray data, although value differences did not match exactly.

#### Differentially expressed genes in insulin sensitive tissues of ZDF rats

In the adipose tissue of ZDF rats, the expression of serine (or cysteine) proteinase inhibitor 1 (PAI-1) in cluster 1, as a known molecular marker of obesity linked to type 2 diabetes, was increased in diabetic ZDF rats at 12 weeks, consistent with previous reports showing that elevated expression levels of PAI-1 was associated with insulin

resistance related to obesity and type 2 diabetes (Bastard *et al.* 2000). Additionally, PAI has been reported to inhibit insulin signaling by competing with  $\alpha\beta 3$  integrin for vitronectin binding (Lopez-Aleman *et al.* 2003), which may explain its role in the development of type 2 diabetes. Furthermore, the expression level of angiotensinogen (AGT) which is involved in blood pressure regulation and adipocyte differentiation, was downregulated in 6-week old pre-diabetic ZDF rats, but upregulated in 12-week old diabetic ZDF rats. Previously, the expression level of AGT in ZDF rats has been reported to be up- or downregulated depending on the age of animals used (Jones *et al.* 1997, Hainault *et al.* 2002). Metallothionein 3, identified in cluster 4, which may have an antioxidant role as a metal-binding and stress-response protein, was downregulated in both pre-diabetic and diabetic ZDF rats. The expression of vitamin D binding protein (DBP), essential for cellular endocytosis and intracellular metabolism of vitamin D,

**Table 1** Genes differentially expressed in adipose tissue of ZDF rats

Accession	Gene Name	Normalized expression (ZDF/ZLC)			Function
		6W	12W	20W	
<b>Cluster 1</b>					
AA901043	EST	0.00	1.75	-1.12	Unclassified
AA900150	NAD <sup>+</sup> -specific isocitrate dehydrogenase b subunit	0.07	1.52	-1.04	Metabolism
AI060068	Fatty acid binding protein3	0.00	1.25	-0.16	Metabolism
AA955115	Carboxylesterase 3	-0.32	1.17	-0.30	Metabolism
AI070183	Nuclear protein 1	-0.24	1.10	-0.04	Transcription
AA955423	Fatty acid binding protein 5, epidermal	-0.13	0.99	ND	Metabolism
AA859385	Vimentin	0.02	0.80	-0.72	Cytoskeleton
AA818904	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	-0.14	0.80	-0.24	Transcription
AA965232	Serine (or cysteine) proteinase inhibitor, member 1	0.09	0.69	-0.68	Endopeptidase
AI044904	Annexin VI	0.08	0.61	-0.79	Calcium binding
AA964578	Calpactin I heavy chain	0.12	0.57	-0.84	Calcium binding
AI070507	Fibromodulin	0.03	0.51	-0.63	Extracellular matrix
AI059907	Chemokine (C-X-C motif) ligand 10	-0.14	0.47	-0.11	Signal transduction
AI059648	Angiotensinogen	-1.03	0.75	0.28	Endopeptidase
AI060165	Lipase, hormone sensitive	-0.55	0.72	ND	Metabolism
AI136048	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	-0.46	0.50	-0.01	Metabolism
AI136365	Sortilin1	-0.39	0.40	-0.03	Transport/Endocytosis
<b>Cluster 2</b>					
AA901405	Protein tyrosine phosphatase, non-receptor type 11	0.11	0.46	-0.95	Signal transduction
AA858569	Neuronatin	0.74	0.66	-0.72	Unclassified
AA963906	RNA binding motif protein 16	0.51	0.02	-0.64	RNA binding
AA996833	Aldolase C, fructose-biphosphate	0.61	-0.39	-0.08	Metabolism
AA900486	ATP citrate lyase	0.44	-0.89	-0.66	Metabolism
AA818413	Clusterin	0.31	-0.58	ND	Apoptosis
<b>Cluster 3</b>					
AA925370	F-spondin	0.04	-0.97	0.79	Extracellular matrix
AA859785	Alcohol dehydrogenase 1	0.03	-0.87	0.50	Metabolism
AA859213	Stearoyl-Coenzyme A desaturase 2	0.14	-0.87	-0.08	Metabolism
AA819161	Desmin	-0.02	-0.65	0.76	Cytoskeleton
AA875489	Voltage-dependent anion channel 1	0.26	-0.76	ND	Ion channel
<b>Cluster 4</b>					
AA818559	EST	-0.88	0.09	0.03	Unclassified
AA925361	FK506-binding protein 1a	-0.64	0.04	0.59	Signal transduction
AA819832	EST	-0.59	0.00	0.86	Unclassified
AA819638	N-acetyltransferase 5	-0.59	0.06	0.77	Unclassified
AA859061	EST	-0.51	-0.01	0.47	Unclassified
AA859231	Tropomyosin 1, alpha	-0.46	-0.18	1.32	Cytoskeleton
AA818706	Group specific component(vitamin D binding protein)	-0.64	-1.08	-0.01	Transport
AA924772	Metallothionein 3	-0.51	-0.55	0.48	Metal ion binding
AI058961	Microtubule-associated protein 1b	-0.68	0.05	0.61	Cytoskeleton
AA875488	EST	-1.20	-0.04	0.84	Unclassified
AA819654	Similar to zinc finger protein (LOC307362)	-0.59	0.04	0.33	Transcription
AA899387	Similar to PD2 protein (LOC361531)	-1.02	-0.08	0.57	Unclassified
AA955420	Guanylate cyclase 1, soluble, beta 3	-0.56	0.24	0.47	Signal transduction
AA819832	Period 1	-0.59	0.00	0.86	Signal transduction

Expression ratios are shown by log<sub>2</sub> scale of normalized mean signal intensities of ZDF rat genes to those of ZLC. ND denotes "non-detectable or low signal-to-noise ratio". W, weeks old.

also was decreased in both pre-diabetic and diabetic ZDF rats. Vitamin D is necessary for maintaining insulin secretion and normal glucose tolerance and influences insulin sensitivity. DBP gene variants have also been shown to be predisposed to type 2 diabetes mellitus (Malecki *et al.* 2002). Therefore, the decreased

expression of DBP is expected to induce insulin resistance. The expression of ATP citrate lyase and stearoyl-coenzyme A desaturase 2 in cluster 2 and 3 respectively, which are involved in triglyceride synthesis, was found to be decreased in 12-week old ZDF rats, whereas lipolytic genes such as hormone sensitive lipase

**Table 2** Genes differentially expressed in skeletal muscle of ZDF rats

Accession	Gene Name	Normalized expression (ZDF/ZLC)			Function
		6W	12W	20W	
<b>Cluster 1</b>					
AA819345	Parvalbumin	-0.02	-1.18	0.86	Calcium binding
AA818892	Pyruvate dehydrogenase	0.07	-1.05	0.48	Metabolism
AA963095	Prohibitin	-0.04	-0.91	0.68	Transcription/cell cycle
AA999182	Solute carrier family 10, member 1	-0.13	-0.66	0.47	Transport
AA817792	EST	-0.01	-0.48	0.42	Unclassified
AA924091	Leprecan-like protein 2	-0.02	-0.31	0.83	Oxidoreductase/metabolism
AA996896	Ubiquitin-conjugating enzyme UBC7	0.39	-0.08	-0.12	ubiquitin-conjugating
AA859043	Triadin 1	0.36	-0.65	0.25	Translation/exocytosis
AA858950	Regulator of G-protein signaling protein 2	0.91	-0.25	0.05	Signal transduction
AA924933	Munc13-4 protein	0.08	-0.43	0.54	Exocytosis
AA899619	Alpha-globin transcription factor CP2	0.07	-0.81	0.29	Transcription
AI028932	Prostaglandin F2 receptor negative regulator	0.01	-0.32	0.19	Signal transduction
<b>Cluster 2</b>					
AA900901	Cyclin F	-2.81	0.06	0.77	Cell cycle
AA900958	SorCS2	-1.78	-0.13	0.98	Transport/Endocytosis
AA818691	Myogenic factor 6	-0.29	0.02	0.57	Transcription
AA817748	EST	-1.11	-0.10	1.29	Unclassified
AA875328	Fas-associated factor 1	-0.79	-0.03	0.79	Apoptosis
AA957538	Vesicular transport protein rvps45	-0.60	-0.17	0.54	Transport
<b>Cluster 3</b>					
AI136093	Fatty acid amide hydrolase	-0.81	0.12	0.07	Metabolism
AA924224	Cytochrome P450, subfamily 11B, polypeptide 2	-0.49	0.24	-0.02	Biosynthesis
AA819300	Squalene epoxidase	-0.74	0.42	-0.06	Oxidoreductase/metabolism
AA818398	Glutathione S-transferase, mu type 3 (Yb3)	-0.49	0.35	-0.08	Transferase
AI072234	p21 (CDKN1A)-activated kinase 1	-0.49	0.29	0.05	Signal transduction
AA859122	EST	-0.39	0.61	0.05	Unclassified
AI136365	Sortilin 1	-0.39	0.40	-0.03	Transport/Endocytosis
AA900185	EST	-0.12	0.77	-0.02	Unclassified
AA925173	Heparanase	-0.11	0.63	-0.30	Extracellular matrix
<b>Cluster 4</b>					
AA818605	EST	1.41	-0.05	-1.23	Unclassified
AA859476	Cofilin 1	0.76	-0.28	-0.18	Cytoskeleton
AA819612	Profilin	0.58	-0.16	-0.06	Cytoskeleton
AA859814	Cystatin B	0.57	-0.06	-0.24	Structure
AA963445	Selenoprotein P, plasma, 1	0.57	-0.20	-0.14	Selenium binding
AA899180	NAD(P)H dehydrogenase, quinone 1	0.87	-1.99	0.02	Oxidoreductase
AA900899	Mitogen-activated protein kinase kinase 6	0.76	-1.32	0.07	Signal transduction
AA924727	Collagen, type 1, alpha 1	1.28	-1.04	-0.05	Extracellular matrix
AA901017	EST	1.12	-1.04	0.12	Unclassified
AA962987	Carbonyl reductase 1	0.97	-0.76	-0.05	Oxidoreductase/metabolism
AA958018	Cd24 antigen	0.79	-0.72	-0.26	Signal transduction
AA819336	Cathepsin H	0.65	-0.32	-0.01	Endopeptidase
AA955881	Fatty acid synthase	0.32	-0.13	0.05	Metabolism
AA900486	ATP citrate lyase	0.44	-0.89	-0.89	Metabolism
<b>Cluster 5</b>					
AA899924	Cd164 antigen	0.18	1.01	-0.31	Signal transduction
AA924388	WD repeat domain 20 isoform 1	-0.17	1.00	-0.84	Meiosis
AA926010	Fatty acid Coenzyme A ligase, long chain 2	0.25	0.77	0.77	Metabolism
AA819897	Synuclein, alpha	0.21	0.25	-0.51	Signal transduction
AI070507	Fibromodulin	-0.10	0.32	-0.50	Extracellular matrix

Expression ratios are shown by log<sub>2</sub> scale of normalized mean signal intensities of ZDF rat genes to those of ZLC. W, weeks old.

and fatty acid binding proteins in cluster 1 were upregulated at 12 weeks.

In the skeletal muscle of ZDF rats, the expression of pyruvate dehydrogenase and NAD(P)H dehydrogenase for glucose oxidation identified in cluster 1 and 4 respectively, were decreased in 12-week old diabetic ZDF rats. Transgellin 3, whose function is related to muscle development, was also downregulated at 12 weeks, suggesting that this gene may play an important role in insulin resistance and type 2 diabetes since insulin resistance is associated with dysregulated myogenic development. The expression of SorCS2 and sortilin genes in cluster 2 and 3 was decreased in 6-week old pre-diabetic ZDF rats. These genes belong to the novel type I transmembrane receptor family containing a common Vps10 domain, a sorting receptor for the carboxypeptidase Y. It has been suggested that sortilin has a cytoplasmic tail sequence homology between sortilin and insulin like growth factor II (Mazella 2001), and co-localizes in intracellular vesicles containing glucose transporter 4 (GLUT4) and translocates into the plasma membrane in response to insulin (Morris *et al.* 1998). SorCS2 has an amino acid sequence similar with sortilin except for the presence of isoleucine/valine rich repeats (Hermeijer *et al.* 1999). Therefore, the down-regulation of Vps10 domain-containing proteins might influence the translocation of GLUT4 in skeletal muscle. Parvalumin and triadin in cluster 1, which play an important role in regulating intracellular calcium concentration through their buffering capacity as high-affinity cytosolic calcium binding proteins (CBPs), were significantly downregulated in ZDF rats 12-weeks old. Regulation of intracellular calcium plays a key role in obesity, insulin resistance, and hypertension. Calcium has been shown to regulate insulin signaling, as increasing levels of calcium impairs insulin signaling, possibly through inhibition of insulin-regulated dephosphorylation, resulting in insulin resistance. Therefore, the reduced expression of CBP in diabetic-stage rats may cause an increase in intracellular calcium and lead to insulin resistance.

In the livers of ZDF rats, glucose-6-phosphatase in cluster 3, involved in gluconeogenesis, was upregulated at 12 weeks, which could contribute to hyperglycemia in ZDF rats. Expression of the mitogen activated protein kinase (MAPK) genes, including stress activated protein kinase 2 and MAPK3 (ERK1), identified in cluster 2, was increased at 6 weeks, and the regulator of G-protein signaling 12 was increased at 12 weeks.

#### **Decreased lipogenic gene expression in diabetic liver, but increased lipogenic gene expression in diabetic skeletal muscle of 12-week old ZDF rats**

We found that lipogenic genes, including ATP citrate lyase and fatty acid coenzyme A ligase (FACL), were

downregulated in the livers of 12-week old diabetic ZDF rats. In order to further confirm the reduced expression of lipogenic genes in the livers of diabetic ZDF rats, we measured the expression of other lipogenic genes including fatty acid binding protein (aP2), peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ), and SREBP1c with genes observed in this study at 6 and 12 weeks of age using real-time RT-PCR. As shown in Fig. 3A, expression levels of aP2, PPAR $\gamma$ , and SREBP1c genes were significantly lower in 12-week old ZDF rats compared with 12-week old ZLC rats, consistent with microarray data. The data also showed that the expression of aP2 and PPAR $\gamma$  was increased from 6 to 12 weeks in both strains, but the increase in ZDF rats was much smaller than in ZLC rats. The expression of SREBP1c and FACL2 was increased from 6 weeks to 12 weeks in ZLC rats, whereas the expression was decreased in ZDF rats. Protein expression levels of PPAR $\gamma$  and SREBP1 were consistent with the results of real-time RT-PCR (Fig. 3C).

In contrast to the decreased lipogenic gene expression found in diabetic livers of ZDF rats, the expression of the lipogenic genes, fatty acid coenzyme A ligase, fatty acid synthase, and ATP citrate lyase, were increased in the skeletal muscle of 12-week old diabetic ZDF rats. Expression of other lipogenic genes including PPAR $\gamma$ , SREBP1c, and stearoyl-coenzyme A desaturase 1 (SCD1) were also measured in the skeletal muscle at 6 and 12 weeks of age with real-time RT-PCR. As shown in Fig. 3B, the expression of SCD1 and FACL2 was significantly higher in the skeletal muscle of 12-week old ZDF rats compared with ZLC rats of the same age. Even though expression of PPAR $\gamma$  and SREBP1c was not shown to be significantly different between ZLC and ZDF rats at 12 weeks of age, the expression of these genes in diabetic ZDF rats was increased from 6 weeks to 12 weeks in contrast to decreases observed in ZLC rats. The protein levels of PPAR $\gamma$  and SREBP1 were also consistent with transcription levels (Fig. 3C).

#### **Reduced expression of antioxidant genes and CYP450 genes in diabetic livers from 12-week old ZDF rats**

Our microarray data also revealed that antioxidant genes, such as hemopexin, glutathione peroxidase 1, superoxide dismutase 1, and glutaredoxin 1 were downregulated in the liver of ZDF rats at 12 weeks of age. Furthermore, the expression of cytochrome P450 (CYP) genes, including CYP4A3, CYP1A2, CYP3A9, and CYP2C39 was also significantly decreased at 12 weeks, although the expression of these genes was increased at 6 weeks. These data strongly suggest that the liver of diabetic ZDF rats at 12 weeks of age has an impaired capacity to manage oxidative stress and some xenobiotics. Therefore, we verified the expression of

**Table 3** Genes differentially expressed in liver of ZDF rats

Accession	Gene Name	Normalized expression (ZDF/ZLC)			Function
		6W	12W	20W	
<b>Cluster 1</b>					
AA859607	Sterol-C4-methyl oxidase-like	-0.04	-0.65	1.58	Metabolism
AA900933	14-3-3 protein epsilon isoform	-0.04	-0.49	0.71	Signal transduction
AA965012	Zinc finger protein 354A	-0.34	-0.02	1.12	Transcription
AA924800	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	-0.27	0.00	1.36	Metabolism
<b>Cluster 2</b>					
AA859455	Sir2-like 3	1.62	-0.15	-0.38	Transcription
AA900766	EST	1.44	-0.13	-0.40	Unclassified
AA998956	Abl-interactor 2	1.24	0.12	-0.49	Signal transduction
AI059997	Stress activated protein kinase alpha II	0.96	-0.02	-0.04	Signal transduction
AA818904	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	0.94	0.01	-0.17	Transcription
AA955475	Ubiquitin-conjugating enzyme E2D 2	0.88	-0.22	-0.19	Ubiquitination
AA925792	Superoxide dismutase 1	0.57	-0.22	-0.01	Oxidoreductase
AA923836	Eukaryotic initiation factor 5 (eIF-5)	0.56	-0.28	-0.06	Translation
AA964788	Glutathione peroxidase 1	0.51	-0.22	-0.39	Oxidoreductase
AA875555	Protein kinase, mitogen activated 3 (ERK1)	0.45	-0.19	0.00	Signal transduction
AA866249	RAB4A, member RAS oncogene family	0.92	-1.67	0.03	Signal transduction
AA818124	Cytochrom P450 15-beta gene	0.79	-1.58	-0.15	Metabolism
AA818680	Ornithine aminotransferase	1.11	-1.47	0.09	Metabolism
AA818180	Murine thymoma viral (v-akt) oncogene homolog 2	0.63	-1.18	-0.03	Signal transduction
AA818043	Cytochrome P450, 2c39	1.66	-1.02	-0.06	Metabolism
AI145654	Protein kinase, cAMP dependent regulatory, type I, alpha	1.01	-1.02	0.02	Signal transduction
AA926256	Neuritin	0.92	-0.97	-0.02	Axonogenesis
AA924591	Cytochrome P450 4A3	0.87	-0.94	0.00	Metabolism
AA900486	ATP citrate lyase	0.44	-0.89	-0.66	Metabolism
AA926359	EST	1.22	-0.88	0.02	Unclassified
AA925350	Insulin-like growth factor binding protein, acid labile subunit	0.55	-0.84	0.02	Signal transduction
AA901404	EST	1.82	-0.83	-0.36	Unclassified
AA926010	Fatty acid Coenzyme A ligase, long chain 2	1.20	-0.78	0.04	Metabolism
AA923919	Cathepsin E	1.09	-0.67	-0.19	Endopeptidase
AA818406	U6 snRNA-associated Sm-like protein LSm6	1.65	-0.63	-0.04	RNA binding
AA875311	Collagen, type V, alpha 2	0.92	-0.60	-0.04	Extracellular matrix
AA859087	Dynactin	1.09	-0.59	-0.10	Cytoskeleton
AA925794	Diazepam binding inhibitor	1.66	-0.55	-0.12	Metabolism
AA819042	Hemopexin	0.91	-0.50	-0.04	Endopeptidase
AA859963	Cytochrome oxidase subunit VIc	0.74	-0.48	-0.08	Metabolism
AI136404	Cytochrome P450 3A9	0.95	-0.43	-0.04	Metabolism
AA859354	G protein gamma-5 subunit	0.60	-0.40	-0.03	Signal transduction
AA818813	Glutaredoxin 1 (thioltransferase)	0.85	-0.40	0.00	Oxidoreductase
AA819611	Insulin-like growth factor binding protein 3	1.08	-0.37	-0.08	Signal transduction
AA955662	Apolipoprotein C-I	0.69	-0.36	-0.12	Metabolism
AA819911	Integrin beta 1	0.72	-0.32	-0.08	Signal transduction
AA819465	Apolipoprotein C-III	0.85	-0.32	-0.12	Metabolism
AA924594	Cytochrome P450, 1a2	0.00	-1.72	0.56	Metabolism
<b>Cluster 3</b>					
AA964628	Glucose-6-phosphatase, catalytic	0.08	0.81	-0.50	Metabolism
AI072234	p21 (CDKN1A)-activated kinase 1	-0.10	0.73	-0.17	Signal transduction
AI144577	Regulator of G-protein signaling 12	-0.16	0.70	0.00	Signal transduction
AA964507	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	-0.08	1.10	-0.24	Transcription
AA924223	Pyroglutamyl-peptidase I	-0.34	0.94	0.00	Proteolysis
AA926047	Vacuolar protein sorting 35	-0.33	0.69	-0.36	Transport/Endocytosis
AA924063	Mitogen activated protein kinase kinase 1	-0.40	0.58	-0.04	Signal transduction
AI059788	Folate hydrolase	-0.37	0.57	0.10	Proteolysis

Table 3 Continued

Accession	Gene Name	Normalized expression (ZDF/ZLC)			Function
		6W	12W	20W	
<b>Cluster 4</b>					
AA859109	EST	-0.80	0.72	-0.01	Unclassified
AI145353	Thyroid hormone receptor alpha	-0.72	0.57	0.05	Signal transduction
AA964978	Phosphofructokinase, muscle	-0.63	0.61	-0.08	Metabolism
AA819072	Sgk	-0.56	0.48	ND	Signal transduction
AA817985	EST	-0.55	0.63	-0.02	Unclassified
AA900733	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	-0.76	0.35	0.22	Signal transduction
AA900759	T-cell death associated gene	-0.76	0.42	0.01	Apoptosis
AA819663	Acyl-CoA:dihydroxyacetonephosphate acyltransferase	-0.93	0.01	0.08	Metabolism
AA924572	Kinesin family member 5B	-1.12	0.60	-0.11	Cytoskeleton
AA901272	EST	-0.75	0.30	0.11	Unclassified
AA924837	Bone morphogenetic protein receptor, type 1A	-0.66	0.39	0.11	Signal transduction
AA819622	Coagulation factor II receptor	-1.19	0.22	0.03	Signal transduction
AA925531	Prolactin-like protein B	-0.66	0.12	0.21	Hormone
AA965187	Lactalbumin, alpha	-0.58	0.23	0.01	Metabolism

Expression ratios are shown by log<sub>2</sub> scale of normalized mean signal intensities of ZDF rat genes to those of ZLC. ND denotes "non-detectable or low signal-to-noise ratio". W, weeks old.

glutathione-related genes and CYP 450 genes in both animals at 6 and 12 weeks of ages with the real-time RT-PCR. As shown in Fig. 4, the expression of glutathione peroxidase 1, superoxide dismutase, and glutaredoxin 1 was decreased in the liver of 12-week old diabetic ZDF rats compared with ZLC rats of the same age, consistent with microarray data. Moreover, the expression of these genes was significantly decreased from 6 weeks to 12 weeks in ZDF rats, whereas the expression was increased in ZLC rats. The expression of CYP4A3, 1A2, 3A9 and 2C39, as shown in Fig. 5, was also decreased in 12-week old ZDF rats.

#### Common genes expressed concurrently in insulin sensitive tissues of ZDF rats

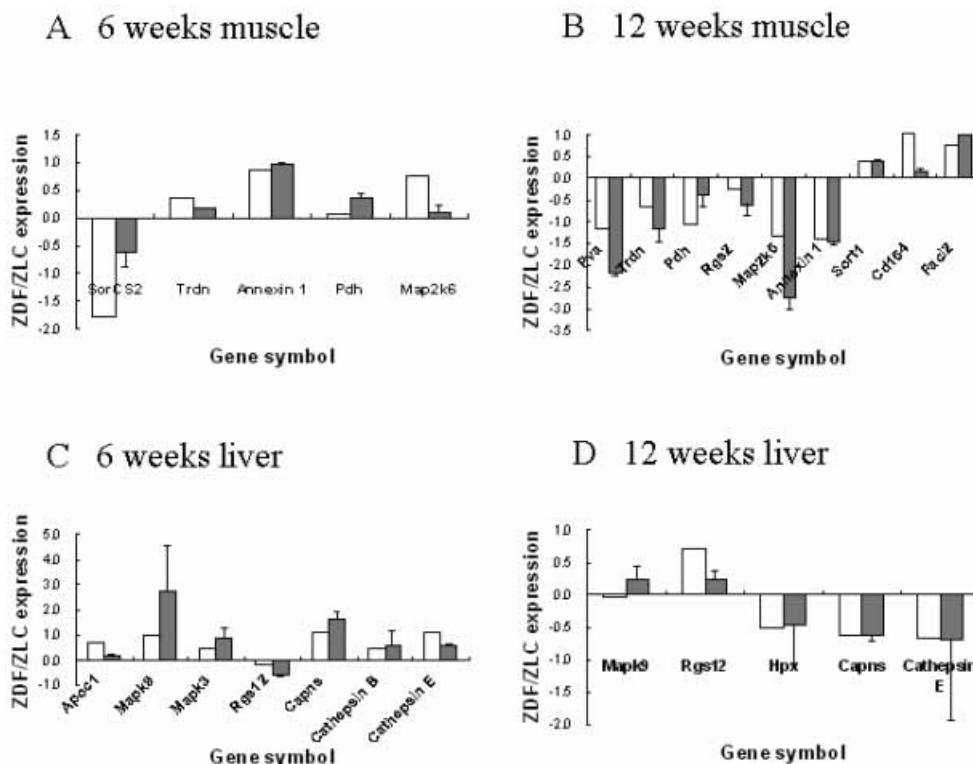
Several common genes expressed concurrently in insulin sensitive tissues of ZDF rats were observed (Table 4). Of them, calpain 4 was downregulated in skeletal muscle and liver of 12-week old rats but in all three tissues of 20-week old diabetic ZDF rats. Annexin1, cathepsin B, and vimentin were upregulated in both pre-diabetic muscle and liver tissue from ZDF rats, but their expression was decreased in diabetic-stage animals. Lumican and enolase 2 were also upregulated in both pre-diabetic adipose and skeletal muscle in ZDF rats, whereas collapsing response mediator protein 1 was significantly downregulated in both pre-diabetic tissue types. Growth associated protein 43, collagen (type 1  $\alpha$ 1), and transgelin 3 were downregulated in both

diabetic adipose and skeletal muscle although the expression was increased in pre-diabetic rats. The expression of protein phosphatase 5 was increased in both diabetic adipose and pre-diabetic liver tissues.

## Discussion

Using microarray technology, it is now possible to examine the transcriptional profile of several thousand genes simultaneously to decipher the pathophysiology of the disease at molecular level. In this study, we assessed the expression profiles of 5000 genes in insulin sensitive tissues from 6-week old pre-diabetic ZDF rats, 12-week old diabetic ZDF rats, and 20-week old late-stage diabetic ZDF rats for a better fundamental understanding of insulin resistance and type 2 diabetes. Collectively, our results suggest a pathophysiological cascade of gene expression in type 2 diabetes in the ZDF model.

Lipolytic gene expression was increased in the adipose tissue of 12-week old diabetic ZDF rats. Increased free fatty acids (FFA) released by adipocytes is a key feature in type 2 diabetes. Since the balance between cellular triglyceride synthesis, FFA re-esterification, and triglyceride hydrolysis determines the amount of FFA released by adipocytes, aberrations in these cellular pathways may contribute to increased FFA production in adipose tissue. Elevated plasma concentrations of FFA can cause insulin resistance by impairing the ability of insulin to stimulate muscle glucose uptake and to inhibit hepatic glucose production (Lewis *et al.* 2002). Lipolysis in



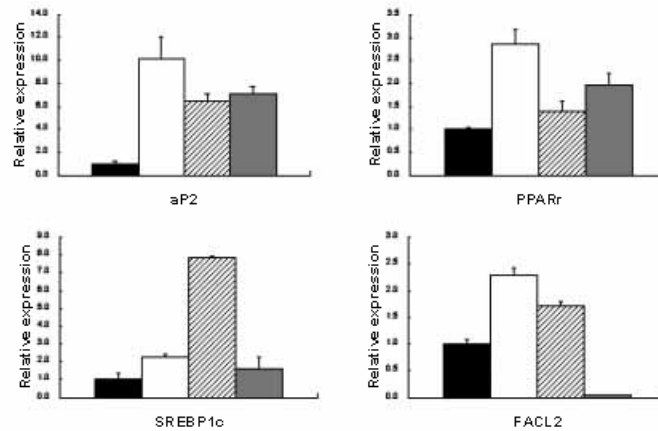
**Figure 2** Relative expression of select transcripts from the skeletal muscle and liver tissue of ZDF or ZLC rats by real-time RT-PCR. Total RNA was extracted from the skeletal muscle or liver of 6- and 12-week old rats, and PCR reactions were carried out in SYBR PCR core reagents. The y-axis is  $\Delta\Delta C_T$  calculated after normalization to 18S rRNA or corresponding relative expression level of ZDF to ZLC rats under the microarray experiments. Grey bars,  $\Delta\Delta C_T$ ; open bars, relative expression level (log<sub>2</sub> scale) under the microarray experiments. Individual  $C_T$  values are representative of means from triplicate measurements. Trdn, triadin 1; Pdh, pyruvate dehydrogenase; Map2k6, mitogen-activated protein kinase kinase 6; Pva, parvalbumin; Rgs2, regulator of G-protein signaling protein 2; Sort1, sortilin; Facl2, fatty acid coenzyme A ligase, long chain 2; Apoc1, apolipoprotein C-I; Mapk9, mitogen activated protein kinase 9; Mapk3, mitogen activated protein kinase 3; Rgs12, regulator of G-protein signaling protein 12; Capns, calpain, small subunit 1; Hpx, hemopexin.

adipose tissue is mediated, in part, via interaction of fatty acid binding protein (FABP) with hormone sensitive lipase (Lewis *et al.* 2002). In our data, ATP citrate lyase and stearoyl-coenzyme A desaturase 2, involved in triglyceride synthesis, were downregulated in adipose tissue of 12-week old ZDF rats, whereas lipolytic genes such as hormone sensitive lipase and FABP were upregulated at 12 weeks. This imbalance may increase the release of FFA from adipose tissue in diabetic ZDF rats and could lead to further insulin resistance and development of type 2 diabetes.

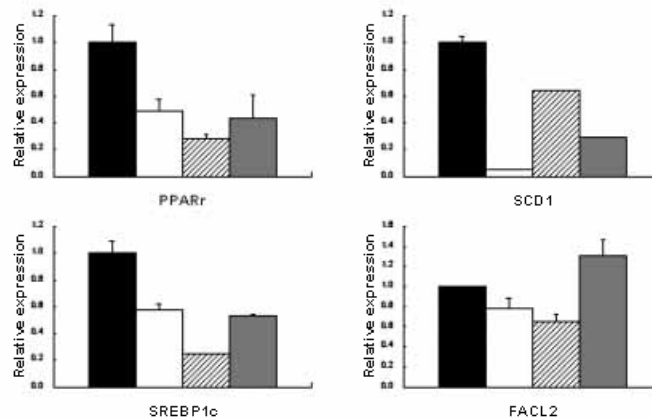
While lipolytic gene expression was increased in the adipose tissues of 12-week old diabetic ZDF rats, the expression of genes involved in lipogenesis was decreased in the liver as well as in the adipose tissue of diabetic ZDF rats at 12 weeks. Reportedly, adipose function, reflected in the expression of lipogenic genes, deteriorates with prolonged obesity (Nadler *et al.* 2000)

and reduced lipogenic adipocytes are associated with increased hepatic lipogenesis, which may be a shift in the lipogenic burden from adipocytes to other organs such as the liver (Diraison *et al.* 2002). However, a failure to increase hepatic lipogenesis in obesity has been shown to contribute to the development of type 2 diabetes (Lan *et al.* 2003). Moreover, increased hepatic lipogenesis is also associated with reduced risk of diabetes in a lipotrophic mouse model (Colombo *et al.* 2003). In our study, the expression of ATP citrate lyase and fatty acid coenzyme A ligase for lipogenesis was significantly downregulated in the liver of 12-week old diabetic ZDF rats although the expression was increased in 6-week old pre-diabetic rats. The expression of other lipogenic genes such as aP2, PPAR $\gamma$ , and SREBP1c also decreased significantly in 12-week old ZDF rats compared with 12-week old ZLC rats, which is shown in Fig. 3A and 3C. Previous reports also showed that lipogenic gene expression in the

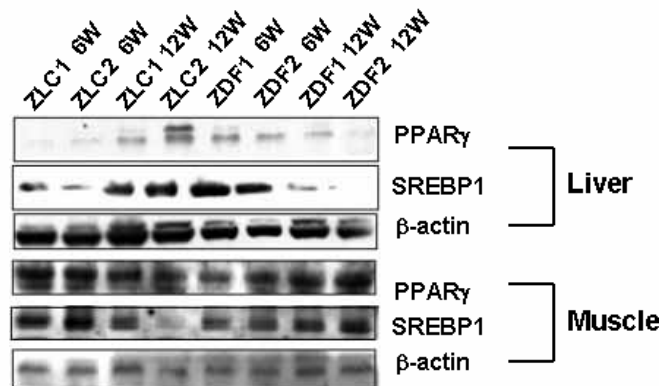
## A Liver



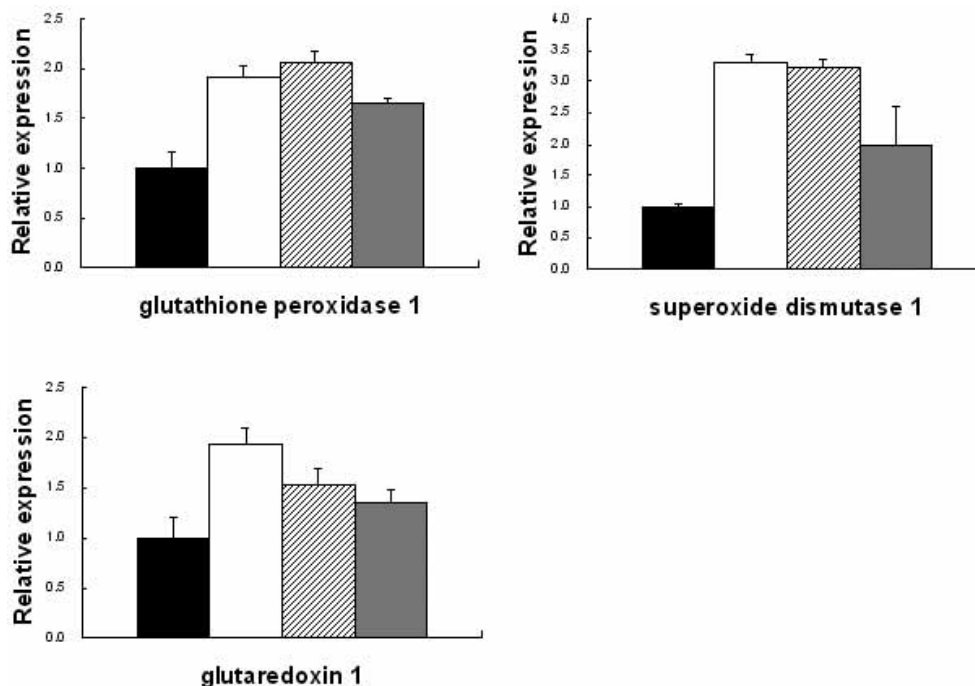
## B Muscle



## C



**Figure 3** Quantitative expression of lipogenic genes in the liver or skeletal muscle at 6 weeks or 12 weeks of age. Total RNA was extracted from the 6- and 12-week old liver (A) or 6- and 12-week-old skeletal muscle (B) of ZDF and ZLC rats, and the PCR reactions were carried out in SYBR PCR core reagents. The y-axis is the relative expression level normalized against the mean expression level ( $2^{-\Delta CT}$  value) in 6-week old ZLC rats. Black bars, 6-week-old ZLC rats; open bars, 12-week-old ZLC rats; hatched bars, 6-week old ZDF rats; grey bars, 12-week old ZDF rats. Liver and muscle tissues of two ZDF and ZLC rats were solubilized and Western blotted with PPAR $\gamma$  or SREBP1 or  $\beta$ -actin antibody (C). aP2, adipocyte fatty acid-binding protein; PPAR $\gamma$ , peroxisome proliferator activated receptor gamma; SREBP1c, sterol regulatory element-binding protein 1c; FAcl2, fatty acid coenzyme A ligase, long chain 2; SCD1, stearoyl-CoA desaturase 1.



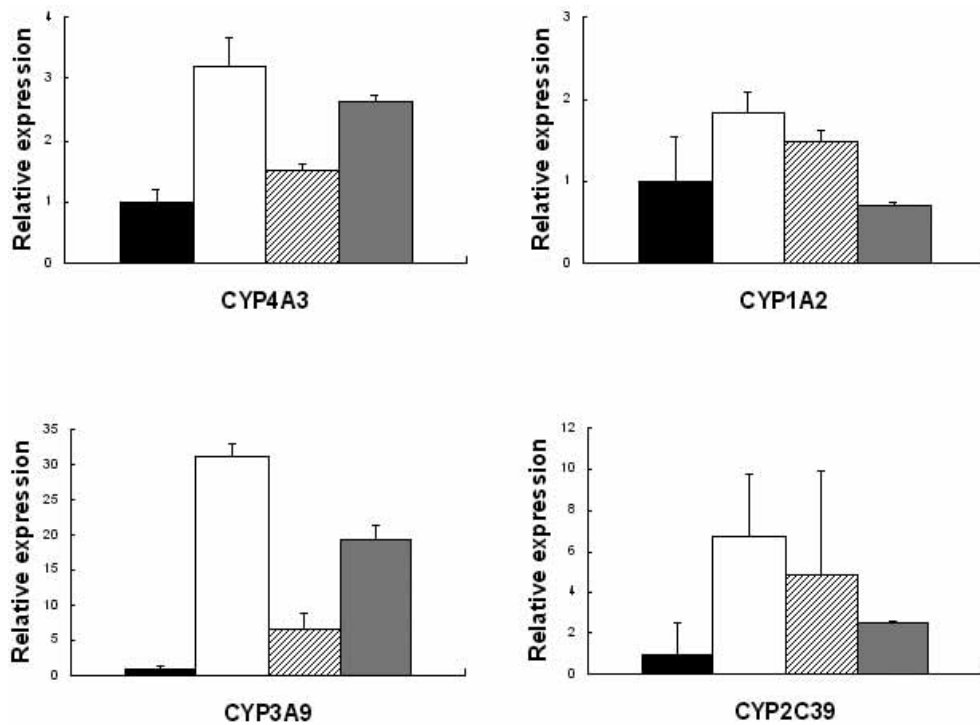
**Figure 4** Quantitative expression of antioxidant genes in the liver at 6 weeks and 12 weeks of age. Total RNA was extracted from the 6-week old liver or 12-week old liver of ZDF and ZLC rats, and the PCR reactions were carried out in SYBR PCR core reagents. The y-axis is the relative expression level normalized against the mean expression level ( $2^{\Delta CT}$  value) in 6-week old ZLC rats. Black bars, 6-week old ZLC rats; open bars, 12-week old ZLC rats; hatched bars, 6-week old ZDF rats; grey bars, 12-week old ZDF rats.

livers of diabetes-susceptible BTBR-ob/ob was decreased compared with diabetes-resistant B6 ob/ob animals, in addition to a dramatic decrease of the expression from 6 weeks (pre-diabetes) to 14 weeks (diabetes) in BTBR-ob/ob (Lan *et al.* 2003). These results suggested that the livers of ZLC rats maintain a high level of hepatic lipogenesis at 12 weeks, whereas lipogenesis in the diabetic livers of ZDF rats was impaired in the transition from the pre-diabetes to diabetes stage. Therefore, the impairment of hepatic lipogenesis in diabetic ZDF rats may contribute to the progression of type 2 diabetes by increasing glucose production and shifting lipogenic burdens to skeletal muscle with deteriorated adipose function.

With impairment of lipogenesis in the livers of 12-week old ZDF rats, we observed an increase in the expression of lipogenic genes, including fatty acid synthase, ATP citrate lyase, and fatty acid coenzyme A ligase in the skeletal muscle of diabetic ZDF rats at 12 weeks of age. Expression of other lipogenic genes, including PPAR $\gamma$ , SREBP1c, and SCD1 also was increased in the skeletal muscle of 12-week old ZDF rats compared with ZLC rats of the same age as shown in Fig. 3B and 3C. Moreover, expression of these lipogenic genes was increased from 6 weeks to 12 weeks, whereas

the expression was decreased in ZLC rats. Potentially, these increases could contribute to muscular triglyceride accumulation, leading to insulin resistance or type 2 diabetes. With intramuscular lipid accumulation, lipid intermediates such as long-chain fatty acyl CoA, diacylglycerol, and ceramide are increased. Increasing lipid intermediates inhibit insulin signaling and insulin-mediated glucose uptake in skeletal muscle (Petersen & Shulman 2002, Schmitz-Peiffer 2002, Lam *et al.* 2003). Therefore, upregulated lipogenesis in the skeletal muscle of diabetic stage animals could contribute to the development of obesity-related diabetes.

Increased hepatic gluconeogenic gene expression in the livers of 12-week old diabetic ZDF rats was observed, as well as reduced gene expression of glucose oxidation in the skeletal muscle and adipose tissue of ZDF rats at 12 weeks. Expression of glucose-6-phosphatase was increased in the liver of ZDF rats at 12 weeks of age, resulting in hyperglycemia. The committed step for glucose oxidation is the conversion of pyruvate to acetyl CoA in the mitochondria by the pyruvate dehydrogenase complex, which then enters the TCA cycle. In our study, expression of pyruvate dehydrogenase (PDX1) and NADP(H) dehydrogenase was decreased in the skeletal muscle of diabetic ZDF



**Figure 5** Quantitative expression of CYP genes in the liver at 6 weeks and 12 weeks of age. Total RNA was extracted from the 6-week or 12-week old liver of ZDF and ZLC rats, and PCR reactions were carried out in SYBR PCR core reagents. The y-axis is the relative expression level normalized against the mean expression level ( $2^{\Delta CT}$  value) in 6-week old ZLC rats. Black bars, 6-week-old ZLC rats; open bars, 12-week-old ZLC rats; hatched bars, 6-week-old ZDF rats; grey bars, 12-week-old ZDF rats.

rats, and voltage-dependent anion channel for oxidative phosphorylation was also decreased in the adipose tissue of diabetic ZDF rats. These results suggest that oxidative metabolism is decreased in both skeletal muscle and adipose tissue of diabetic ZDF rats at 12 weeks of age.

The expression of antioxidant genes was downregulated in adipose and liver tissues of 12-week old diabetic ZDF rats. More recently, studies have linked reactive oxygen species (ROS) production and oxidative stress to insulin resistance (Evans *et al.* 2002). *In vitro*, ROS and oxidative stress led to the activation of multiple serine kinase cascades, which phosphorylates insulin-signaling substrates including the insulin receptor (IR) and insulin receptor substrate (IRS)-1, and inhibits tyrosine phosphorylation (Evans *et al.* 2002). Therefore, antioxidants could prevent the activation of these serine kinases induced by ROS and oxidative stress. In our study, metallothionein 3, which plays a role as an antioxidant protecting ROS, as well as a detoxifying antidote for metals, was downregulated in the adipose tissues of ZDF rats at both ages. Hemopexin (Hpx), which acts as an antioxidant through its strong heme binding, was also downregulated in liver of diabetic ZDF rats progressing from pre-diabetes to diabetes. Importantly, the expres-

sion of glutathione-related defense genes including glutathione peroxidase 1, superoxide dismutase 1, and glutaredoxin 1 was increased in the livers of 6-week old pre-diabetic ZDF rats which may be responsible for defenses against oxidative stress, but was significantly reduced at 12 weeks (diabetes stage). We verified the expression of these genes in both animals at each age with real-time RT-PCR. As shown in Fig. 4, expression levels in ZLC rats were increased from 6 weeks to 12 weeks, whereas expression in ZDF rats was significantly decreased. Therefore, the reduced expression of these antioxidant genes in diabetic livers may partly account for insulin resistance and development of type 2 diabetes.

Although the expression of cytochrome P450 (CYP) was increased in 6-week old pre-diabetic ZDF rats, CYP450 expression was significantly reduced in the livers of 12-week old diabetic ZDF rats. Cytochrome P450 constitutes a superfamily of heme-proteins that play important roles in the detoxification of numerous xenobiotics, as well as endogenous compounds including steroids, fatty acids, and prostaglandins. In various disease states, including diabetes, obesity, and inflammation and infection, the expression of hepatic

**Table 4** Concurrent expressed genes in adipose tissue, skeletal muscle and liver of ZDF rats

	<u>Tissue</u>	<u>6W, NE</u>	<u>12W, NE</u>	<u>20W, NE</u>	<u>Description</u>
<b>Accession</b>					
<b>Muscle &amp; liver</b>					
AA875382	muscle	-0.17	0.47	-0.41	calbindin 1
	liver	1.07	-0.64	0.02	
AA819452	muscle	0.72	-0.27	-0.09	transgelin
	liver	0.54	-0.08	-0.25	
AA963225	muscle	0.66	-0.16	-0.07	cathepsin B
	liver	0.47	-0.16	-0.04	
AA963445	muscle	0.57	-0.20	-0.14	selenoprotein P, plasma, 1
	liver	0.65	-0.51	0.03	
AA859385	muscle	0.68	-0.63	-0.23	vimentin
	liver	0.87	-0.20	-0.07	
AA924402	muscle	0.48	-0.29	-0.54	general transcription factor IIa, 2
	liver	0.98	-0.54	-0.26	
AA964960	muscle	0.87	-1.39	-0.26	annexin 1
	liver	0.82	-0.98	-0.78	
<b>Muscle &amp; fat</b>					
AA925443	muscle	-0.39	0.63	-0.17	cellular retinoic acid binding protein 2
	fat	-0.70	0.00	0.94	
AA924564	muscle	-1.98	-0.01	0.63	mitochondrial ribosomal protein L11
	fat	-1.12	-0.04	1.24	
AA998065	muscle	-2.14	0.03	0.90	collapsin response mediator protein 1
	fat	-1.72	-0.07	0.61	
AA866389	muscle	0.56	-0.43	0.01	lumican
	fat	0.53	-0.08	-0.53	
AA924727	muscle	1.28	-1.04	-0.05	collagen, type 1, alpha 1
	fat	0.71	-0.54	-0.65	
AA997308	muscle	0.45	-0.27	-0.08	enolase 2, gamma
	fat	0.45	-0.35	-0.24	
AI043649	muscle	1.28	-0.85	-0.18	growth associated protein 43
	fat	0.71	-0.72	-0.17	
AI044424	muscle	0.71	-1.16	-0.23	transgelin 3
	fat	0.68	-0.62	-0.28	
<b>Fat &amp; liver</b>					
AA818443	fat	-0.01	-0.25	0.65	dipeptidylpeptidase 4
	liver	0.80	-0.76	0.00	
AI072330	fat	0.13	0.38	-0.52	lactate dehydrogenase A
	liver	1.31	-0.05	-0.38	
AA925017	fat	0.05	0.65	-0.58	chemokine (C-X-C motif) ligand 11
	liver	0.72	-0.55	-0.01	
AA956187	fat	0.06	0.60	-1.05	protein phosphatase V
	liver	0.67	-0.43	0.00	
AI145101	fat	-0.03	0.60	-0.80	hydroxyacyl glutathione hydrolase
	liver	0.70	-0.83	0.00	
<b>Muscle &amp; liver &amp; fat</b>					
AA900053	muscle	0.97	-0.32	-0.14	calpain, small subunit 1
	fat	0.30	0.41	-1.24	
	liver	1.11	-0.63	-0.32	
AA925675	muscle	1.06	-0.85	-0.06	collagen, type III, alpha 1
	fat	0.72	-0.05	-0.38	
	liver	0.96	-0.52	-0.15	
AA875221	muscle	0.98	-0.07	-1.07	isocitrate dehydrogenase 3, gamma
	fat	-0.20	1.10	-0.12	
	liver	1.14	-0.99	0.04	

Expression ratios are shown by log<sub>2</sub> scale of normalized mean signal intensities of ZDF rat genes to those of ZLC. NE denotes "Normalized Expression(ZDF/ZLC)". W, weeks old.

P450 in the liver changes markedly. In streptozotocin-induced diabetes, hepatic expression of CYP2B, CYP2E1, and CYP1A2 was increased, but expression of CYP2C11, CYP2C13, CYP2A2, and CYP3A2 was decreased (Thummel & Schenkman 1990). In New Zealand obese mice, which exhibit a polygenic syndrome of obesity, insulin resistance, dyslipidemia, and hypertension, the expression of CYP2B9, CYP3A16, and CYP4A14 was increased markedly in livers from diabetic mice, but only slightly increased in insulin resistant mice. In contrast, expression of CYP2C22, CYP2C29 and CYP2C40 was reduced in diabetic, but not affected in insulin resistant mice. Furthermore, expression of CYP1A2 and CYP7B1 was reduced in both diabetic and insulin resistant mice (Pass *et al.* 2002). In our present study, expression of CYP4A3, CYP3A9, CYP2C39, and CYP1A2 was increased in the livers of pre-diabetic ZDF rats, but was significantly reduced in diabetic livers, which was confirmed by real-time RT-PCR (Fig. 5). Although these alterations correlated with changes in serum free fatty acid levels and seem to be mediated by PPAR- $\alpha$ , the reduction of these CYP enzymes in diabetic livers of ZDF rats may contribute to the development of type 2 diabetes due to a reduced capacity for xenobiotic detoxification.

Analyzing microarray data, we found that the expression of MAP serine/threonine kinase pathway genes was up- or downregulated depending on the tissue type. Stress activated protein kinase (SAPK) alpha 2 (referred to as JNK/SAPK) and extracellular signal regulated kinase1 (ERK-1) were upregulated in the livers of 6-week old pre-diabetic ZDF rats, but mitogen activated protein kinase kinase 6 (MKK6), which activates p38 MAPK, was downregulated in the skeletal muscle of ZDF rats at 12 weeks of age. It was reported that constitutive activation of MEK1-ERK downregulates the expression of GLUT4, IR, IRS-1 and IRS-2, resulting in marked impairment of insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2, docking of the p85 $\alpha$  regulatory subunit of PI3K, and PI3K activation (Hirosumi *et al.* 2002). Furthermore, the activation of MKK7-JNK pathway also suppressed tyrosine phosphorylation of IRS-1 or IRS-2, which in turn, suppressed activation of PI3K and Akt. It was also reported that JNK/SAPK activity is abnormally elevated in obesity (Hirosumi *et al.* 2002). Taken together, activation of ERK, JNK can induce insulin resistance in pre-diabetic livers. Previous reports demonstrated that the p38 MAPK inhibitor, SB203580, prevented insulin-stimulated glucose uptake in 3T3-L1 adipocyte and L6 muscle cells (Sweeney *et al.* 1999) and that alpha lipoic acid activated glucose uptake via a p38 MAPK-dependent pathway (Konrad *et al.* 2001). Therefore, downregulation of MKK6, upstream of p38 MAPK, may lead to decreased insulin-stimulated glucose transporter in diabetic skeletal muscle.

We also observed several common genes regulated differentially in insulin sensitive tissues including annexin, regulator of G-protein signaling (RGS), and calpain 4. Annexin 1 was upregulated in the skeletal muscle and livers of pre-diabetic ZDF rats. The annexin superfamily, which consists of at least 11 different, abundant, and ubiquitously expressed proteins, reportedly play important roles in the regulation of insulin secretion and the effects of insulin on its target tissues by inhibition of IR tyrosine phosphorylation (Melki *et al.* 1994). Furthermore, it was reported that the annexin 1 gene harbors variants that increase the risk of type 2 diabetes (Lindgren *et al.* 2001). All together, upregulation of annexin 1 in pre-diabetic skeletal muscle and liver tissue may induce insulin resistance and contribute to the development of type 2 diabetes. Additionally, expression of the regulator of G-protein signaling (RGS) gene, which is essential for catecholamine and insulin action, was increased in pre-diabetic skeletal muscle and diabetic liver tissue in ZDF rats. The G protein signaling pathway is regulated by the interplay between receptor-catalyzed activation and inhibitory RGS proteins. RGS proteins acts like a GTPase-activating protein (GAP) specific for Gi and Gq class G protein alpha subunit, and plays a crucial role in processes responsible for shutting off G-protein-mediated cell responses in eukaryotes (Ishii & Kurachi 2003). Recent observations have found possible linkages between heterotrimeric G-proteins and insulin signaling (Luft 1997). Reportedly, the G alpha-q/11 protein is required for insulin-induced glucose transport in 3T3-L1 adipocyte in PI3-kinase dependent or independent signal transduction pathways (Imamura *et al.* 1999). Therefore, the upregulation of RGS in insulin sensitive tissues could attenuate insulin stimulated GLUT4 translocation by inhibiting signaling by GTP binding protein, and lead to insulin resistance in the pre-diabetic and diabetic stages. Another cysteine proteinase, calpain 4, was downregulated in the skeletal muscle and liver of 12-week old diabetic ZDF rats, although the expression was increased in 6-week old pre-diabetic ZDF rats. Recently, one member of the calpain family, calpain 10, was identified by positional cloning as a susceptibility gene for type 2 diabetes. Genetic studies have shown that variations in the calpain gene are associated with type 2 diabetes (Horikawa *et al.* 2000). Furthermore, low levels of calpain 10 mRNA in skeletal muscle has also been reportedly associated with insulin resistance (Baier *et al.* 2000). Treatment of muscle strips with the calpain inhibitor, ALLM and E-64-d resulted in a significant reduction in the rate of insulin-stimulated glucose uptake and the incorporation of glucose into glycogen (Sreenan *et al.* 2001). Therefore, the reduction of calpain 4 gene expression from pre-diabetes to diabetes in insulin sensitive tissues may also attenuate the insulin-stimulated glucose uptake and

play an important role in the development of type 2 diabetes.

In summary, we assessed global transcriptional profiles of insulin sensitive tissues of ZDF male rats at a pre-diabetic and diabetic stage. This approach, coupled with current metabolic knowledge, provided several candidate genes and molecular pathology of insulin resistance and the development of type 2 diabetes.

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