A Nutrigenomic Framework to Identify Time-Resolving Responses of Hepatic Genes in Diet-Induced Obese Mice

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Obesity and its related complications have emerged as global health problems; however, the pathophysiological mechanism of obesity is still not fully understood. In this study, C57BL/6J mice were fed a normal (ND) or high-fat diet (HFD) for 0, 2, 4, 6, 8, 12, 20, and 24 weeks and the time course was systemically analyzed specifically for the hepatic transcriptome profile. Genes that were differentially expressed in the HFD-fed mice were clustered into 49 clusters and further classified into 8 different expression patterns: long-term up-regulated (pattern 1), long-term down-regulated (pattern 2), early up-regulated (pattern 3), early down-regulated (pattern 4), late up-regulated (pattern 5), late down-regulated (pattern 6), early up-regulated and late down-regulated (pattern 7), and early down-regulated and late up-regulated (pattern 8) HFD-responsive genes. Within each pattern, genes related with inflammation, insulin resistance, and lipid metabolism were extracted, and then, a protein-protein interaction network was generated. The pattern specific sub-network was as follows: pattern 1, cellular assembly and organization, and immunological disease, pattern 2, lipid metabolism, pattern 3, gene expression and inflammatory response, pattern 4, cell signaling, pattern 5, lipid metabolism, molecular transport, and small molecule biochemistry, pattern 6, protein synthesis and cell-to cell signaling and interaction and pattern 7, cell-to cell signaling, cellular growth and proliferation, and cell death. For pattern 8, no significant sub-networks were identified. Taken together, this suggests that genes involved in regulating gene expression and inflammatory response are up-regulated whereas genes involved in lipid metabolism and protein synthesis are down-regulated during diet-induced obesity development.

INTRODUCTION

Obesity is defined as an accumulation of excess body fat in the body and its prevalence is rapidly increasing throughout the world. Besides physical, social, and psychological disadvantages, obesity can lead to serious health problems including insulin resistance (IR), type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) and certain types of cancers (Haslam and James, 2005). Although there are various causes of obesity including genetic susceptibility, endocrine disorders, medical side-effects, psychiatric disorders, or cerebropathy, the main etiology of obesity is attributed to a combination of excessive energy intake and reduced energy expenditure such as physical activity (Bleich et al., 2008).

High fat diet (HFD) has known to induce obesity and related complications in rodents and other animals. Particularly, C57BL/6J mouse is a well-known animal model used for a diet-induced obesity (DIO) since this strain develops visceral fatness, IR, hyperinsulinemia and hyperlipidemia those seen in humans upon HFD feeding (Lin et al., 2000; Petro et al., 2004; Rossmeisl et al., 2003; Surwit et al., 1995; Van Heek et al., 1997).

The liver is one of the key organs in the development of obesity and related disorders. Excessive food (energy) consumption increases the supply of oxidizable substrates to the liver that are utilized for energy synthesis or for the production of biosynthetic compounds. This increases the hepatic content of total cholesterol and triglycerides (TG), which eventually leads to fatty liver and liver cirrhosis. IR, reduced insulin sensitivity of insulin target tissues, also occurs in the liver during obesity development. As consequences of IR, the action of insulin such as glucose uptake, inhibition of gluconeogenesis, and inhibition of lipolysis are all decreased in the liver (Schenk et al., 2008). IR also reduces the uptake of circulating lipids and increases the hydrolysis of stored TG in adipocytes. Under this condition,

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adipocytes increase the release of non-esterified fatty acids (NEFAs) into the blood, following a reduction of muscle glucose uptake and an increase of hepatic glucose production, which contribute to a gradual increase of blood glucose levels (Sell et al., 2006). Recently, obesity has been referred to as a low-grade systemic inflammatory disease. Proinflammatory cytokines (e.g. Tnf α , Il6, Crp, Resistin, Serpine1) and NEFAs produced in adipocytes result in impaired insulin action/endothelial function, hyperinsulinemia, glucose intolerance, and other complications in both obese animals and humans (Fain, 2006; Fried et al., 1998; Xu et al., 2003). Especially, high intake of saturated and trans-FAs potentiates the promotion of obesity by playing a considerable role in modulating lipid metabolism, IR, and inflammation (Matsuzawa-Nagata et al., 2008; Rocha and Libby, 2009).

With advances in genome-wide microarray technology, it is now possible to gain deep insight into the hepatic transcriptional changes in DIO. The differences in the expression of genes involved in lipid metabolism, IR, and inflammation have also been studied by cross-sectional microarray analysis in various murine DIO models. However, understanding of time-resolved gene expression changes during prolonged HFD feeding is of crucial importance for developing strategies to prevent and treat irreversible disease characteristics. Radonjic et al. studied hepatic gene expression associated with short and long-term exposure to excess dietary fat during 16 weeks in ApoE3Leiden mice (Radonjic et al., 2009). They found that the expression of inflammatory/immune pathways was activated in the early phase (day 1 to week 1) and was repressed in the late phase (week 8 to week 16) of the experimental period. Similarly, a time-resolved system analysis of gene expression and metabolite levels in liver, white adipose tissues, and muscles was performed to elucidate the pathogenesis of IR in HFD fed ApoE3 Leiden mice (Kleemann et al., 2010). IR first manifested in the liver (at week 6) and then in white adipose tissue (WAT) (at week 12) whereas white skeletal muscles remained insulin-sensitive. HFD also evoked an early hepatic inflammatory response which then gradually declined. Inflammation, however, increased over time in WAT and it gradually was suppressed in skeletal muscles with HFD. Since these studies were undertaken with unusually HFDs for 12 and 16 weeks in ApoE3L transgenic mice, we investigated the changes of temporal hepatic gene expression with physiologically relevant and prolonged (24 weeks) HFD-feeding which compensate potential adaptation responses to increased energy intake in C57BL/6J mice compared to age-matched normal diet (ND)-fed controls (Do et al., 2011). In contrast to previous reports (Kleemann et al., 2010; Radonjic et al., 2009), we found that inflammation associated genes were elevated consistently over 24 weeks and IR with elevated plasma insulin and impaired glucose tolerance was observed after 16 weeks of HFD feeding while fasting plasma glucose was elevated even after 20 weeks. Hepatic lipid accumulation and elevation of plasma total cholesterol was significant long before IR induction. Although differences in diet, animals, and duration of experiment gives different results, it is clear that genes involved in inflammation, IR, and lipid metabolism are most significantly changed by HFD. In this study, we therefore just focused on the genes involved in inflammation, IR, and lipid metabolism from the past microarray analysis data and further analyzed time-dependent transcriptional changes of these genes to develop strategies to prevent and treat irreversible disease characteristics.

MATERIALS AND METHODS

Data analysis

We obtained microarray data from previous studies (Do et al., 2011) and analyzed time-dependent transcriptional changes of genes involved in inflammation, IR, and lipid metabolism as shown in Fig. 1.

Clustering of hepatic gene transcripts

For clustering of hepatic gene transcripts, the Self-Organizing Map (SOM) algorithm (Quackenbush, 2001) was applied. We used Genowiz (Ocimum Biosolutions, India) with the SOM tool to arrange all data samples.

Construction of HFD responsive hepatic gene networks

HFD responsive hepatic gene networks related to inflammation, IR, and lipid metabolism were constructed by the Michigan Molecular Interactions (MiMI) plug-in (Gao et al., 2009) for Cytoscape (<http://www.cytoscape.org>). The MiMI plug-in for Cytoscape enables one to connect to the MiMI database and construct the interactions. We used the protein-protein interactions from the biomolecular interaction network database (BIND) (Bader et al., 2003), the database of interacting proteins (DIP) (Salwinski et al., 2004), the biological general repository for interaction datasets (BioGRID) (Stark et al., 2011), IntAct (Kerrien et al., 2012), the kyoto encyclopedia of genes and genomes (KEGG) (Kanehisa et al., 2012), the molecular interaction database (MINT) (Licata et al., 2012), PubMed, and the reactome (Croft et al., 2011) in MiMI.

Gene set enrichment analysis

To identify the biological functions associated with the HFD responsive genes over the entire expression patterns, the DAVID Functional Annotation Clustering tool (<http://david.abcc.ncifcrf.gov/>) (Huang da et al., 2009a; 2009b) was used. Gene set enrichment analysis (GSEA) was performed using Gene Ontology (GO) (biological process, molecular function, and cellular component) and pathways (BIOCARTA and KEGG pathway) according to a False Discovery Rate (FDR) < 5% and a Benjamin and Hochberg adjusted p-value < 0.05.

Analysis of networks and pathways

Analyses of networks, pathways, and their functions were done with the Ingenuity Pathway Analysis (IPA; Ingenuity Systems, USA) (Viswanathan et al., 2008; Werner, 2008). The gene lists containing gene identifiers and corresponding expression values for each HFD responsive hepatic gene network related to inflammation, IR, and lipid metabolism were uploaded to IPA and these gene lists were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base, a repository of biological interactions and functional annotations created from millions of individually modeled relationships between proteins, genes, complexes, cells, tissues, metabolites, drugs, and diseases, and networks and pathways were generated and visualized based on the connectivity of the genes. The analysis of networks and pathways specified the most significant biological functions, canonical pathways, toxicological functions and diseases related to the genes in the network and pathway and Fischer's exact test was used to calculate a p-value to determine the probability.

Fig. 1. Flow chart of experiments and data analysis.

RESULTS

Statistical analysis of hepatic gene expression changes by HFD and clustering of the transcripts

The changes in HFD-induced hepatic gene expression over 24 weeks compared to ND were first evaluated at each of the time points. By applying a fold change of gene expression and statistical cutoff with a False Discovery Rate (FDR) < 5%, a Benjamin and Hochberg adjusted p-value < 0.05, and a log fold change > 1, we identified 939 and 4,136 differentially expressed genes by HFD, which were up- or down-regulated by fold changes and T-test comparison, respectively, across all of the time-points (Table 1).

To classify and group the data based on similar expression patterns by function of time, hepatic gene transcripts that were differentially expressed in the HFD-fed mice were clustered into 49 clusters with the SOM algorithm (Fig. 2). The clusters were then further classified into 8 expression patterns to identify time-resolving responses of hepatic gene expression by HFD feeding as follows (Table 2); long-term up-regulated (pattern 1),

long-term down-regulated (pattern 2), early up-regulated (pattern 3), early down-regulated (pattern 4), late up-regulated (pattern 5), late down-regulated (pattern 6), early up-regulated and late down-regulated (pattern 7), and lastly, early down-regulated and late up-regulated (pattern 8) HFD-responsive genes. At this point, we determined that 2, 4, 6, and 8 weeks were the early stage and the last 4 periods across all of the time-points, 12, 16, 20, and 24 weeks, were the late stage of obesity development.

HFD responsive hepatic gene networks related to inflammation, IR, and lipid metabolism

It is important to see how these genes functionally interact to access biological features of the genes. We therefore constructed a molecular interaction network with the MiMI plug-in for Cytoscape and characterized the functional annotation of the network using GO and pathway classification analysis for each expression pattern based on a FDR < 5% and a Benjamin and Hochberg adjusted p-value < 0.05 (Supplementary data 1). The network was, however, too big to analyze the biological

Table 1. Number of differentially expressed hepatic gene transcripts per time-point which were up- or down-regulated by fold changes and T-test comparison in C57BL/6J mice fed high-fat diet during 24 week time-course

	Number of DEG based on HFD vs. ND by fold change		Number of DEG based on HFD vs. ND by T-test (FDR q-value < 0.1)
	Up	Down	
2 week	94	16	202
4 week	65	35	1
6 week	93	25	2,298
8 week	165	30	253
12 week	64	30	180
16 week	65	49	1
20 week	59	40	79
24week	77	32	1,122
All time point	682	257	4,136
Total	939		4,136

Fig. 2. Clusters of hepatic gene transcripts that were differentially expressed in C57BL/6J mice fed a high-fat diet over 24 weeks by Self-Organizing Map (SOM). x-axis, duration of time; y-axis, log gene change.

functions. We therefore focused on genes related to inflammation, IR, and lipid metabolism since these are the three major features of obesity. Filtered genes by manual curation included 65 inflammation-, 6 IR-, and 53 lipid metabolism-related genes (Supplementary data 2). In addition, 19 genes related to inflammation and lipid metabolism, 17 genes related to IR and lipid metabolism, and 793 genes related to inflammation, IR, and lipid metabolism were identified. This limited set of HFD responsive genes of interest was used for all downstream functional analyses. In addition, to include prior biological know-

ledge, this limited gene set was used for GSEA by the DAVID Functional Annotation Clustering tool.

As a result of the enriched functions (Supplementary data 3), regulation of phosphorylation, positive regulation of response to stimulus, activation of immune response, immune system development, positive regulation of immune response, positive regulation of cell differentiation, and leukocyte activation related functions were uniquely enriched in the network of pattern 1. In this case, some immune related functions were uniquely observed. In the case of the pattern 2 transcripts network, some

Table 2. Expression patterns of high-fat diet responsive hepatic gene clusters

Pattern No.	Expression patterns	Clusters
1	Long-term up-regulated HFD-responsive genes	C08, C14, C31, C37, C39, C41
2	Long-term down-regulated HFD-responsive genes	C15, C27, C28, C42
3	Early up-regulated HFD-responsive genes	C01*, C04, C18, C22, C23*, C38*
4	Early down-regulated HFD-responsive genes	C07 [#] , C17, C29 [#] , C34, C40 [#] , C43, C44 [#] , C47, C48
5	Late up-regulated HFD-responsive genes	C02, C03, C07 [#] , C13, C19, C29 [#] , C35, C40 [#] , C44 [#]
6	Late down-regulated HFD-responsive genes	C01*, C21, C23*, C32, C38*, C49
7	Early up-regulated and late down-regulated HFD-responsive genes	C01*, C23*, C38*
8	Early down-regulated and late up-regulated HFD-responsive genes	C07 [#] , C29 [#] , C40 [#] , C44 [#]

*Pattern 7 (early up-regulated and late down-regulated HFD-responsive genes) also clustered into pattern 3 (early up-regulated HFD-responsive genes) and pattern 6 (late down-regulated HFD-responsive genes).

[#]Pattern 8 (early down-regulated and late up-regulated HFD-responsive genes) also clustered into pattern 4 (early down-regulated HFD-responsive genes) and pattern 5 (late up-regulated HFD-responsive genes).

metabolic functions were uniquely enriched, such as hexose catabolic process, glucose catabolic process, glycolysis, and glucose metabolic process. Some signal transduction related functions, such as regulation of Ras protein signal transduction and regulation of small GTPase mediated signal transduction, were uniquely enriched in the pattern3 transcripts network. Ras protein signal transduction was uniquely enriched in the pattern 5 transcripts network and cellular response to stress was uniquely enriched in the pattern 6 transcripts network. Positive regulation of apoptosis was uniquely enriched in the pattern 7 transcripts network and coenzyme metabolic process was uniquely enriched in the pattern 8 transcripts network.

Supplementary data 4 shows the enriched pathways of the HFD responsive hepatic gene transcripts networks related with inflammation, IR, and lipid metabolism. Thrombin signaling and protease-activated receptors, phospholipase C signaling pathway, activation of Src by protein-tyrosine phosphatase alpha, and fibrinolysis pathway from BIOCARTA and complement and coagulation cascades, leukocyte transendothelial migration, and TGF-beta signaling pathway from KEGG were uniquely enriched in the network of pattern 1. In the case of the pattern 2 transcripts network, regulation of BAD phosphorylation from BIOCARTA and glycolysis/gluconeogenesis, type II diabetes mellitus, and PPAR signaling pathway from KEGG were uniquely enriched. In this case, some metabolic and metabolic disorder related pathways were uniquely observed. CTL mediated immune response against target cells, signaling pathway from G-protein families, and NF-kB signaling pathway from BIOCARTA were uniquely enriched in the pattern 3 transcripts network and TGF beta signaling pathway from BIOCARTA was uniquely enriched in the pattern 4 transcripts network. In the case of the pattern 7 transcripts network, IL2 signaling pathway from BIOCARTA was uniquely enriched.

Network analysis of HFD-responsive genes related to inflammation, IR, and lipid metabolism

HFD responsive hepatic gene networks related to inflammation,

IR, and lipid metabolism were then re-constructed for each expression pattern (Supplementary data 5), and then, to further investigate the biological connectivity between the HFD responsive hepatic genes, the set of genes for each expression pattern was used as an input for the network analysis within the IPA suite. The top 5 networks with the highest significance score and their associated biological functions are listed in Table 3. To restrict the size and facilitate the clarity of the resulting network, we focused on the most relevant sub-network associated with HFD-induced obesity.

The sub-network for pattern 1 consisted of genes related to cellular assembly and organization, cancer, and immunological disease (Fig. 3A). Especially, the expression of Gdi and Hcls1 was up-regulated throughout the entire experimental period. The analysis identified that the sub-network for pattern 2 consisted of genes related to cellular assembly and organization, cellular function and maintenance, and lipid metabolism (Fig. 3B). Acadl, Acd, Apoa4, Bag4, Cyb5r3, Cyp8b1, and Ppargc1a were significantly down-regulated from weeks 2 to 24 in HFD fed mice compared to the ND fed controls. In this sub-network, the results show that several major regulators of the cellular response to HFD appeared as network hub nodes. Pparg is a regulator of lipid, fatty acid, and cholesterol metabolism and Nfkb is a regulator of the immune response. The sub-network for pattern 3 consisted of genes related to gene expression, antimicrobial response, and inflammatory response. As shown in Fig. 3C, Irak1, Irf7, Myd88, and Vps29 were early up-regulated in HFD fed mice. The results show that Ikb and Ikb family member Ikbkb and Nfkb modulator were observed as the network hub nodes. In the case of the pattern 4, the sub-network consisted of genes related to cell signaling, cellular assembly and organization, and cellular function and maintenance (Fig. 3D). Rhot2 and Sh3gl1 were down-regulated by week 4 in HFD-fed mice. In the sub-network for pattern 5, the sub-network consisted of genes related to lipid metabolism, molecular transport, and small molecule biochemistry (Fig. 3E). Acad9, Acads, Acadvl, Apob, and Got2 were up-regulated after 12 weeks of

Fig. 3. Significant sub-network of unique protein-protein interactions network for each HFD responsive hepatic gene expression clusters related with inflammation, IR, and lipid metabolism. (A) Significant sub-network of unique protein-protein interactions network for pattern 1 (long-term up-regulated HFD-responsive genes) associated with cellular assembly and organization, cancer, and immunological disease. (B) Significant sub-network of unique protein-protein interactions network for pattern 2 (long-term down-regulated HFD-responsive genes) associated with cellular assembly and organization, cellular function and maintenance, and lipid metabolism. (C) Significant sub-network of unique protein-protein interactions network for pattern 3 (early up-regulated HFD-responsive genes) associated with gene expression, antimicrobial response, and inflammatory response. (D) Significant sub-network of unique protein-protein interactions network for pattern 4 (early down-regulated HFD-responsive genes) associated with cell signalling, cellular assembly and organization, and cellular function and maintenance. (E) Significant sub-network of unique protein-protein interactions network for pattern 5 (late up-regulated HFD-responsive genes) associated with lipid metabolism, molecular transport, and small molecule biochemistry. (F) Significant sub-network of unique protein-protein interactions network for pattern 6 (late down-regulated HFD-responsive genes) associated with RNA post-transcriptional modification, protein synthesis, and cell-to-cell signalling and interaction. (G) Significant sub-network of unique protein-protein interactions network for pattern 7 (early up-regulated and late down-regulated HFD-responsive genes) associated with cell-to-cell signalling and interaction, cellular growth and proliferation, and cell death.

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HFD feeding. Particularly interesting is the sub-network consisted of major regulators of cellular response to HFD; Pparg is a regulator of lipid, fatty acid, and cholesterol metabolism and Nfkb is a regulator of the immune response, appearing as the network hub nodes similar to the sub-network for pattern 2. Figure 3F shows that the sub-network for pattern 6 consisted of genes related to RNA post-transcriptional modification, protein synthesis, and cell-to-cell signaling and interaction. Eef1b2, Rpsa, and Rps3a were down-regulated after 12 weeks of HFD feeding. Finally, the sub-network for pattern 7 consisted of genes related to cell-to-cell signaling and interaction, cellular growth and proliferation, and cell death (Fig. 3G). Rfc2, Rpl29, and Rpsa were up-regulated until 8 weeks of HFD feeding, and then, the expression was down-regulated after that. The expression of Txnip was, however, up-regulated by week 16 in HFD-fed mice and then down-regulated. Although this does not exactly fit into our classification (by 8 weeks as an early stage of obesity development), we included Txnip in pattern 7 since we more focused on turn-around of expression change. In the case of pattern 8, no significant sub-networks were identified from the analysis of networks and pathways using the IPA suite.

The top biological functions, canonical pathways, and toxicological functions associated with the selected sub-networks are shown in Supplementary data 6, 7, and 8.

DISCUSSION

Excess dietary fat intake without noticeable physical activity is one of the major causes of obesity. Since obesity and its detri-

mental complications, including IR, T2DM, CVD, and certain types of cancers, progress chronically, it is of crucial importance to dissect the temporal changes of gene expression during prolonged HFD feeding to develop a prevention or treatment strategy for the diseases.

In this study, we fed C57BL/6J mice with either ND or HFD for 0, 2, 4, 6, 8, 12, 20, and 24 weeks, and the time course was systemically analyzed specifically for the hepatic transcriptome profile. This animal model shares many similarities with human obesity, IR, and T2DM when fed HFD over a prolonged period of time (Winzell and Ahren, 2004). It is generally known that C57BL/6J mice become obese and develop IR after continuous feeding of HFD for 8-10 weeks (Surwit et al., 1995; 1998).

The global changes in the hepatic transcriptome in gene clustering revealed that noticeable genetic responses to excess dietary fat proceeded in two phases: early and late. We therefore classified 49 clusters into 8 different expression patterns according to the expression changes in these two phases and focused on genes involved in inflammation, IR, and lipid metabolism which are three major features of DIO. IPA analysis of a comprehensive PPI network with the genes involved in inflammation, IR, and lipid metabolism for each expression pattern retrieved the top 5 sub-networks and we further analyzed one of the sub-networks which appears to be the most significant to obesity development.

For pattern 1, long-term up-regulated HFD responsive genes, Gdi and Hcls1 were up-regulated during the 24 weeks of HFD feeding compared to the ND fed controls in the sub-network of PPI interactions associated with cellular assembly and organi-

Table 3. Associated network functions of unique protein-protein interactions network for each HFD responsive hepatic gene expression clusters related with inflammation, IR, and lipid metabolism

Expression patterns	Associated network functions
Pattern 1 (Long-term up-regulated)	Drug metabolism, glutathione depletion in liver, hematological system development and function DNA replication, recombination, and repair, cellular assembly and organization, cell cycle Molecular transport, RNA trafficking, connective tissue development and function Cellular assembly and organization, cancer, immunological disease Gene expression, RNA post-transcriptional modification, cell signaling
Pattern 2 (Long-term down-regulated)	Gene expression, RNA post-transcriptional modification, protein synthesis Cardiovascular disease, genetic disorder, neurological disease Genetic disorder, metabolic disease, cancer Cellular assembly and organization, cellular function and maintenance, lipid metabolism Lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism
Pattern 3 (Early up-regulated)	DNA replication, recombination, and repair, cancer, infection mechanism Gene expression, antigen presentation, cardiovascular system development and function Cell morphology, cell death, cell-mediated immune response Gene expression, antimicrobial response, inflammatory response Cell signaling, cancer, reproductive system disease
Pattern 4 (Early down-regulated)	Cardiovascular disease, genetic disorder, neurological disease DNA replication, recombination, and repair, energy production, nucleic acid metabolism Cell signaling, cellular assembly and organization, cellular function and maintenance Gene expression, RNA post-transcriptional modification, inflammatory response RNA post-transcriptional modification, protein synthesis, gene expression
Pattern 5 (Late up-regulated)	DNA replication, recombination, and repair, cellular assembly and organization, cell cycle Lipid metabolism, molecular transport, small molecule biochemistry DNA replication, recombination, and repair, nucleic acid metabolism, small molecule biochemistry Cellular development, cell morphology, skeletal and muscular system development and function Cardiovascular disease, cell cycle, cancer
Pattern 6 (Late down-regulated)	DNA replication, recombination, and repair, energy production, nucleic acid metabolism Gene expression, RNA post-transcriptional modification, infection mechanism RNA post-transcriptional modification, protein synthesis, cell-to-cell signaling and interaction Cellular function and maintenance, molecular transport, cell morphology RNA post-transcriptional modification, DNA replication, recombination, and repair, molecular transport
Pattern 7 (Early up- and late-regulated)	Cell-to-cell signaling and interaction, cellular growth and proliferation, cell death
Pattern 8 (Early down- and late-regulated)	-

zation, cancer, and immunological disease. It has been shown that Gdi1 and Gdi2 exhibit a similar distribution to Slc2a4 and Rab10 at the trans-Golgi network in insulin sensitive tissues including adipocytes and muscles (Chen et al., 2009). Moreover, overexpression of either Gdi or Gdi retention on adipocyte membranes strongly inhibits insulin-stimulated translocation of Slc2a4 onto the plasma membrane in 3T3-L1 adipocytes (Chen et al., 2009; Chinni et al., 1998). This suggests that increases of Gdi expression is one of the causes of HFD-induced IR in the liver.

For pattern 2, long-term down-regulated HFD responsive genes, *Acadl*, *Acd*, *Apoa4*, *Bag4*, *Cyb5r3*, *Cyp8b1*, and *Ppargc1a* were significantly down-regulated from week 2 to 24 in HFD fed mice compared to the ND fed controls in the sub-network of PPI interactions associated with cellular assembly and organization, cellular function and maintenance, and lipid metabolism. *Acadl* catalyzes the first step of fatty acid β -oxidation in the mitochondria. An *Acadl* knockout mice study showed that a deficiency in the *Acadl* gene caused a fatty liver and hepatic IR in mice (Zhang et al., 2007). *Apoa4* is an apolipoprotein com-

ponent of chylomicron particles and modulates the efficiency of enterocyte and hepatic transcellular lipid transport. Its synthesis is confined to the intestine but hepatic synthesis also occurs in mice and rats. *Apoa4* gene deletion impaired the ability of *Apoa4* knockout mice to gain weight and increased adipose tissue mass (Simon et al., 2011). Both *Cyb5r3* and *Cyp8b1* (also known as sterol 12- α -hydroxylase) are endoplasmic reticulum membrane proteins and function in cholesterol biosynthesis and drug metabolism. A study on the Lean and Fat polygenic obesity mouse models developed from the same base population by long-term (over 60 generations) divergent selection for low or high body fat % revealed that *Cyp8b1* gene expression was higher in the obesity-resistance Lean line than the obesity-susceptible Fat line (Simonic et al., 2011). *Ppargc1a* is a transcription coactivator interacting with a broad range of transcription factors that are involved in adaptive thermogenesis, mitochondrial biogenesis, glucose/fatty acid metabolism, peripheral circadian clock, fiber type switching in skeletal muscle, and heart development (Liang and Ward, 2006; Liu and Lin, 2011). The expression of *Ppargc1a* in the liver is induced by

Table 4. Summary for protein-protein interactions network that common between each HFD responsive gene transcripts network and unique protein-protein interactions network for each HFD responsive gene transcripts network.

Expression patterns	Significant genes	Significant molecular and cellular functions
Pattern 1 (Long-term up-regulated)	Gdi, Hcls1	Cellular assembly & organization Cancer Immunological disease
Pattern 2 (Long-term down-regulated)	Acadl, Acd, Apoa4, Bag4, Cyb5r3, Cyp8b1, Ppargc1a	Lipid metabolism (cholesterol synthesis) Cellular assembly & organization Cellular function & maintenance
Pattern 3 (Early up-regulated)	Irak1, Irf7, Myd88, Vps29	Inflammatory & immunity Gene expression Antimicrobial response
Pattern 4 (Early down-regulated)	Rhot2, Sh3gl1	Cell signalling Cellular assembly & organization Cellular function & maintenance
Pattern 5 (Late up-regulated)	Acad9, Acads, Acadvl, Apob, Got2	Lipid metabolism (FA β -oxidation) Nuclear transport Small molecule biochemistry
Pattern 6 (Late down-regulated)	Eef1b2, Rpsa, Rps3a	Protein synthesis RNA post-transcriptional modification Cell-to-cell signalling & interaction
Pattern 7 (Early up- and late-regulated)	Rfc2, Rpl29, Rpsa, Txnip	Cellular energy production Protein synthesis Cell-to-cell signalling & interaction Cellular growth & proliferation
Pattern 8 (Early down- and late-regulated)	-	-

starvation and regulates fasting adaptation, including gluconeogenesis, fatty acid β -oxidation, ketogenesis, heme biosynthesis, and bile acid homeostasis (Yoon et al., 2001). In accordance with this, forced overexpression of Ppargc1a in primary hepatocytes drove gluconeogenic gene expression (Yoon et al., 2001) whereas a knockdown of Ppargc1a by short interfering RNA in the mouse liver significantly reduced Pepck and G-6-Pase (Koo et al., 2004). Ppargc1a knockout mice also exhibited fasting hypoglycemia due to impaired gluconeogenic gene expression (Leone et al., 2005; Lin et al., 2004). In contrast, Ppargc1a is expressed at very low levels in the liver under fed conditions (Puigserver et al., 1998) which can lead to decreased lipid oxidation, IR, obesity, and T2DM (Miura et al., 2003).

For pattern 3, early up-regulated HFD responsive genes, Irak1, Irf7, Myd88, and Vps29 were up-regulated from 2 to 8 weeks of HFD feeding in the sub-network of PPI interactions associated with gene expression, antimicrobial response, and inflammatory response. Irak1 plays a key role in the LPS-mediated Tlr4 pathway (Singh and Li, 2012). Lack of Irak1 caused significantly lower lipid peroxidation and nitrite levels, as well as pro-inflammatory mediators (Singh and Li, 2012). Leptin increases the expression level of Irak1. Macrophages that lack Irak1 show a significantly lower expression of Il6 following LPS or LPS plus leptin stimulation (Vaughan and Li, 2010). On the other hand, it has been shown that mouse Pelle-like kinase (mPLK, homolog of human Irs1) directly phosphorylates Irs1 at Ser 24 which degrades Irs1 and can lead to IR. The activity is in fact increased by Tnfa or Il1 treatment of primary adipose cells (Kim et al., 2005). This suggests a cross-talk between inflammation and IR. Myd88 is a universal adapter protein used

by all Toll like receptors (TLRs) except for Tlr3 and Il1 receptor signaling, which is involved in the activation of inflammatory pathways. Central nervous system-restricted Myd88 deficient mice are protected from HFD-induced weight gain and leptin resistance (Kleinridders et al., 2009) while Yokoyama et al. (2012) recently found that HFD-fed Myd88 deficient mice exhibited a dramatic increase of Stearoyl-CoA desaturase 1 (Scd1) which is a rate-limiting enzyme in monounsaturated fatty acid biosynthesis in the liver and a severe diabetic phenotype.

For pattern 4, early down-regulated HFD responsive genes, cell signaling related genes including Rhot2 and Sh3gl1 were down-regulated by week 4 in HFD-fed mice.

For pattern 5, late up-regulated HFD responsive genes, Acad9, Acads, Acadvl, Apob, and Got2, were up-regulated after 12 weeks of HFD feeding in the sub-network of PPI interactions associated with lipid metabolism, molecular transport, and small molecule biochemistry. Acads function to catalyze the initial step of FA β -oxidation in the mitochondria and are categorized into short-, medium-, or long-chain Acads based on their specificity for the chain length of the target FAs. Acad9 is a novel Acad that is highly homologous to human Acadvl and has maximal activity with long-chain unsaturated acyl-CoAs as the substrate including C16:1-, C18:1-, C18:2-, and C22:6-CoA. In general, decreased mitochondrial FA oxidation has been implicated in T2DM and obesity; paradoxically however, Acadvl deficient mice were protected from HFD-induced obesity and insulin resistance (Zhang et al., 2010). Interestingly, either lean or obese DIO rats fed a low-fat diet or HFD, respectively, showed reduced liver mRNA expression of Acadvl (Ji and Friedman, 2007). The Apob gene encodes two isoforms of Apob proteins,

ApoB48 and ApoB100. The first is synthesized in the small intestine and is the primary apolipoprotein of the chylomicrons. The latter is synthesized in the liver and is the main apolipoprotein of the low-density lipoproteins (LDL). Especially ApoB100 in LDL acts as a ligand for LDL receptors and transports plasma cholesterol to the liver and other tissue cells, which can cause a fatty liver, atherosclerosis, and heart disease. An association between hypercholesterolemia and increased ApoB protein levels has been well established (Gaffney et al., 2002; Veerkamp et al., 2002). ApoB is also an apolipoprotein in very low density of lipoprotein (VLDL) which encapsulates TGs and cholesterol into circulation from the liver. Therefore, the inhibition of ApoB by siRNA or gene targeting oligonucleotide can induce liver steatosis. In fact, one of ten patients administered mipomersen, an antisense APOB synthesis inhibitor, developed mild steatosis (Visser et al., 2010). In addition, mice harboring a genetic defect of ApoB exhibited hepatic TG accumulation (Lin et al., 2002).

For pattern 6, late down-regulated HFD responsive genes, protein synthesis related genes including Eef1b2, Rpsa, and Rps3a were down-regulated after 12 weeks of HFD feeding.

For pattern 7, early-up and late down-regulated HFD responsive genes, a significant sub-network of PPI interactions was associated with cell-to-cell signaling and interaction, cellular growth and proliferation, and cell death. Rfc2, Rpl29, and Rpsa were up-regulated until 8 weeks of HFD feeding and their expression was down-regulated thereafter. The expression of Txnip was up-regulated by 16 weeks in HFD-fed mice and then down-regulated. Txnip is expressed in various tissues including the pancreas, hypothalamus, and adipose tissues and plays an important role in nutrient sensing and in the regulation of energy metabolism. Glucose and diabetes upregulate β -cell Txnip expression and Txnip overexpression induces β -cell apoptosis. In contrast, a Txnip knockout or nonsense mutation promotes β -cell survival and prevents streptozotocin- and obesity-induced diabetes (Chen et al., 2008; Xu et al., 2012). Hyperglycemic ob/ob mice also expressed more Txnip in adipose tissue compared to wild-type mice (Koenen et al., 2011) while the reduction of Txnip expression in ob/ob mice dramatically improved hyperglycemia and glucose intolerance (Yoshihara et al., 2010). Moreover, these mice exhibited enhanced insulin sensitivity in both adipose tissue and skeletal muscles (Chutkow et al., 2010; Yoshihara et al., 2010). Txnip expression in mediobasal hypothalamus was induced by acute nutrient excess and in mouse models of obesity and diabetes (Blouet and Schwartz, 2011). Down-regulation of this Txnip expression, however, prevented DIO and IR. Accordingly, it is thought that early up-regulation of Txnip in our study reflects sensing of nutrient excess and IR while late down-regulation of Txnip is a result of compensation for β -cell disruption.

In summary, inflammation and immunity related gene expression was early up-regulated and the increased expression lasted throughout the experiment (Table 4). Lipid metabolism, especially cholesterol synthesis, related gene expression was down-regulated throughout the experimental period. However, genes involved in FA β -oxidation was late up-regulated. Genes involved in ATP synthesis was up-regulated at the early stage of obesity development while protein synthesis associated genes were down-regulated at the later time points. For the identified significant genes in each expression pattern, it certainly will be necessary to verify the expression of the genes and to further study the functional significance during DIO development. Nevertheless, the presented research findings provide a way to use high-throughput dataset analyses to generate

testable hypotheses on the development of DIO prevention strategies.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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