Mol. Cells *31*, 239-246, March 31, 2011
DOI/10.1007/s10059-011-0026-6

Molecules and Cells



# Identification of the Target Proteins of Rosiglitazone in 3T3-L1 Adipocytes through Proteomic Analysis of Cytosolic and Secreted Proteins

**Hyun-Ho Hwang[1,2], Pyong-Gon Moon[1,2], Jeong-Eun Lee[1,2], Jung-Guk Kim[3], Wan Lee[4], Sung-Ho Ryu[5], and Moon-Chang Baek[1,2,*]**

**Rosiglitazone, one of the thiazolidinedione (TZD), is an oral antidiabetic drug that activates a gamma isoform of peroxisome proliferator-activated receptor (PPARγ). To identify target proteins induced by rosiglitazone in adipocytes, we first performed simultaneous in-depth proteomic profiling of cytosolic proteins and secreted proteins (secretome) from 3T3-L1 adipocytes using a label-free quantification method with nano-UPLC MS/MS. In total, we identified 646 proteins from 3T3-L1 adipocytes, of which 172 and 162 proteins were upregulated and downregulated >1.5-fold, respectively, in rosiglitazone-treated cells, as compared to controls. Some differentially expressed proteins in particular, including fatty acid translocase (FAT)/CD36, fatty acid binding protein, lipoprotein lipase, acetyl CoA acyltransferase, carnitine O-palmitoyltransferase 2, sterol carrier protein, adiponectin, and phosphoenolpyruvate carboxykinase could explain the current action mechanism of TZDs. Furthermore, this study is the first to report on two potential target proteins of rosiglitazone, such as adenomatosis polyposis coli 2 (APC2), and eukaryotic translation initiation factor 5A-1 (eIF5A) related to apoptosis and cell division. Our data clearly suggest that in-depth proteomic approaches using cytosolic and secreted proteins are important and necessary for identification of drug targets at the protein level.**

## INTRODUCTION

Global prevalence of obesity and type 2 diabetes has increased epidemically during recent decades, and will impose an accelerating burden on society and health regulators (American Diabetes Association, 2008; Buschemeyer and Freedland, 2007; Ebrahimpour et al., 2006). In insulin resistance, insulin-responsive cells are not able to mount a normal response to a given insulin stimulation due to impaired function; this condition precedes development of type 2 diabetes (Yki-Jarvinen, 1994). For insulin resistance patients, insulin sensitivity can be improved by treatment with the insulin-sensitizing drugs known as thiazolidinediones (TZD) (Nolan et al., 1994). The proposed action mechanism of the adipocyte-mediated glucose-lowering effect of TZDs is the 'fatty acid steal' hypothesis from blood and the release of adipokines, such as adiponectin, into blood (Yki-Jarvinen, 2004).

TZDs can bind to a gamma isoform of peroxisome proliferator-activated receptor (PPARγ), a key adipogenic transcription factor involved in physiological functions such as energy balance, liquid metabolism, and glucose control (Rangwala and Lazar, 2004). PPARγ in particular is highly expressed in adipose tissue, where it plays a central role in adipose tissue function. Most of the known target genes that are transcriptionally activated by PPARγ belong to the pathway of lipid storage, glucose homeostasis, and its transcriptional regulation of a number of genes, including genes encoding lipoprotein lipase (LPL), fatty acid transporter protein (FATP), adipocyte fatty acid-binding protein aP2, acyl-CoA synthetase (ACS), phosphoenolpyruvate carboxy kinase (PEPCK), and malic enzyme (ME) (Berger and Moller, 2002; Castelein et al., 1994; Schoon-jans et al., 1995; Tontonoz et al., 1994a; 1995). Furthermore, PPARγ can mediate differentiation of preadipocytes to adipocytes (Schoonjans et al., 1996; Tontonoz et al., 1994a; 1994b) and increase the number of small adipocytes and fat mass in animals and humans (Boden et al., 2003; de Souza et al., 2001). Adipocytes, compared to preadipocytes, have higher numbers of glucose transporters and insulin receptors; therefore, they have a higher capacity for fatty acid uptake and lipid storage (Gregoire et al., 1998).

This mechanism has generally been accepted; however other actions of rosiglitazone may exist. The increased number of small adipocytes caused by TZD treatment might be a result of formation of new adipocytes, as well as the disappearance/shrinkage of existing mature adipocytes by inducing adipocyte apoptosis (Okuno et al., 1998; Yamauchi et al., 2001). Body weight gain is due to increased adiposity. However, according to one report, this might also be a result of fluid retention (Fonseca, 2003). As described above, the action mechanism of insulin sensitization by TZDs has been studied; how-

[1]Department of Molecular Medicine, Kyungpook National University, Daegu 700-422, Korea, [2]Cell and Matrix Biology Research Institute, Kyungpook National University, Daegu 700-422, Korea, [3]Department of Internal Medicine, Kyungpook National University Hospital, Daegu, 700-721, Korea, [4]College of Medicine, Dongguk University, Gyeongju 780-714, Korea, [5]Pohang University of Science and Technology, Pohang 790-784, Korea
*Correspondence: mcbaek@knu.ac.kr

Received September 7, 2010; revised November 27, 2010; accepted December 6, 2010; published online January 6, 2011

**Keywords:** 3T3-L1 adipocytes, nano-UPLC MS/MS, proteomic analysis, rosiglitazone

ever, it is not fully understood.

The endocrine functions of the adipose organ have been widely studied and, in particular, adipocytes communicate with many organs (Halberg et al., 2008). Studies in the late 1980s demonstrated that adipocytes can secrete a number of factors and that secretion of some of these factors is affected by metabolic dysregulation (Cook et al., 1987; Flier et al., 1987). Adipose tissue-derived factors are generally referred to as adipokines. These adipokines influence a number of important systemic phenomena and interact in the process with a large number of different organ systems. Several adipokines have shown a critical role in diabetes. Among them, resistin (Kim et al., 2001; Steppan et al., 2001) is a member of the resistin-like molecule hormone family. Initial findings have demonstrated that resistin expression is reduced by thiazolidinedione treatment and is increased in obesity. Studies using mouse models have determined that resistin antagonizes insulin action in the liver (Rajala et al., 2003).

To identify potential protein target of rosiglitazone, several proteomic approaches has been reported recently (Ahmed et al., 2010; Wang et al., 2007). These groups have used traditional proteomic approach using 2 D SDS-PAGE gel and MALDI-TOF method for cytosolic proteins only. Based on this study, generally high abundant proteins shown on SDS-PAGE have been identified but many important proteins well known as the targets of TZD pathway, e.g. fatty acid translocase (FAT)/CD36, lipoprotein lipase and adiponectin, has not been identified. Therefore, it will be a good approach to use nano-LC MS/MS with label-free quantification to identify potential targets of rosiglitazone with low abundance.

In this study, we have focused on in-depth proteome profiling of proteins, including cytosolic as well as secreted proteins from differentiated 3T3-L1 after treatment with rosiglitazone. 3T3-L1 cells are an excellent model system for *in vitro* obesity study. Rosiglitazone was treated after differentiation induction of 3T3-L1 preadipocytes. Each cytosolic proteome and secretome was trypsin-digested and identified by nano-UPLC MS/MS and quantified by label free software known as "IDENTITY". Based on these results, we identified many known or unknown proteins that are expressed differentially by rosiglitazone, and are related to fatty acid, glucose, energy metabolism, and other functions. Therefore, this result assists in the understanding of various action mechanisms of rosiglitazone at the protein level and adds further insight into possible molecular determinants of metabolic disorders including obesity and type II diabetes.

## MATERIALS AND METHODS

### Cell and reagents

Mouse 3T3-L1 preadipocytes were kindly donated as a gift by Young-Bum Kim of Harvard Medical School. Cell culture media was purchased from Hyclon (USA). HPLC-grade ACN was purchased from Merck (Germany). TFA was purchased from Pierce Biotechnology Inc. (USA). Formic acid was purchased from Fluka (Neu-Ulm, Germany). Sequencing grade-modified trypsin was a product of Promega (USA). DTT, Iodoaceteamide, Dexametasone, and insulin were purchased from Sigma Chemical Co. (USA). HPLC grade water was purchased from J.T. Baker (Phillispsburg, USA).

### 3T3-L1 differentiation and sample preparation

Mouse 3T3-L1 preadipocytes were differentiated as previously described (Green and Kehinde, 1974). Cells were cultured in DMEM/high glucose with 10% fetal bovine serum in 5% $CO_2$ at 37°C. Cells were grown to confluence in DMEM with 10% fetal bovine serum in 5% $CO_2$. Two days after reaching confluence (day 0), the cells were induced into differentiation by changing of the medium to DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methyxanthine, 0.25 μM dexamethasone, and 1 μM insulin. After 48 h (day 2), the medium was replaced with DMEM/high glucose supplemented with 10% fetal bovine serum and 1 μM insulin. The medium was changed every second day. On day 6, differentiated cells, grown in DMEM/high glucose supplemented with 10% fetal calf serum, were treated with 0.5 μM rosiglitazone and 1 μM insulin. In parallel, control cells were cultured in the same medium with 1 μM insulin. Differentiation was monitored by the visual appearance of fat droplets in the cells.

### Sample preparation

On day 10 differentiated 3T3-L1 adipocytes were washed with extreme care 5 times with PBS and starved in serum and phenol red free DMEM for 4 h in order to obtain secretome. After starvation, cells were washed again twice with PBS. After 15 h, supernatants (secretome) were harvested and the cells (cytosolic proteins) were harvested in lysis buffer. Supernatants were subsequently filtrated using a 0.22 μm syringe filter. Filtrated secretome was stored at -80°C after lyophilization. Cytosolic proteins were desalted by HLB cartridge (Waters co). Proteins from cytosol and secretome were reduced by 10 mM DTT at 60°C for 30 min and alkylated in 55 mM iodoacetamide (IAA) at room temperature in darkness. Proteolytic digestion of cytosolic and secreted proteins was conducted with 100 ng/μl trypsin dissolved in 25 mM ammonium bicarbonate (ABC), and incubated at 37°C overnight. Peptides were extracted from the gel three times, using 50 μl of 25 mM ABC and 100 μl of 5% formic acid/25 mM ABC/50% ACN in water. Peptides extracted in four steps were combined together, concentrated by Speed-Vac to dry, and subjected to LC-MS/MS analysis.

### Nano-UPLC and Q-TOF MS analysis

0.5 μg of tryptic peptides mixed with 50 fmol of tryptic enolase (yeast enolase; Swiss-prot accession no. P00924) was injected for each analysis. Liquid chromatography and mass spectrometry consisted of a Waters Acquity UPLC system connected to a Waters Q-TOF Premier mass spectrometer (Waters, USA), operated in $MS^E$ mode. Capillary voltage and cone voltage were set to 3,500 and 30 V, respectively. The Q-TOF premier MS acquisition rate was set to 1 s with a 0.1 s interscan delay. The MS and MS/MS scan range was from 50 to 1,990 m/z. Collision energy was conducted using a ramping collision energy from 15 to 35eV. All analyses were acquired using the lock spray to ensure accuracy and reproducibility; GFP was used as the lock mass at a concentration of 300 pmol/uL and a flow rate of 300 nl/min, generating an [M + 2H] ion at 785.8425.

Chromatographic separation was performed on a 75 mm × 250 mm ACQUITY™ 1.7 μm C18 column (Waters, USA) using a ACQUITY™ Ultra Performance. The two mobile phases were as follows: phase A; water/formic acid (99.9/0.1 v/v) and phase B; formic acid/acetonitrile (0.1/99.9 v/v): Gradient conditions were as follows: 3% B at 0.0 min ramped to 40% B after 5 min, ramped to 40% B after 180 min, held at 80% until 195 min, returned to 0% B at 196 min, and held at 0% B until 210 min to refresh the column. The gradient duration was 180 min at a flow rate of 300 nl/min. Mixture samples were diluted in 40 μl of mobile phase A and 5 μl each was injected three times onto the column

### Protein identification and relative quantification analysis

The acquired MS and MS/MS data were aligned by MassLynx

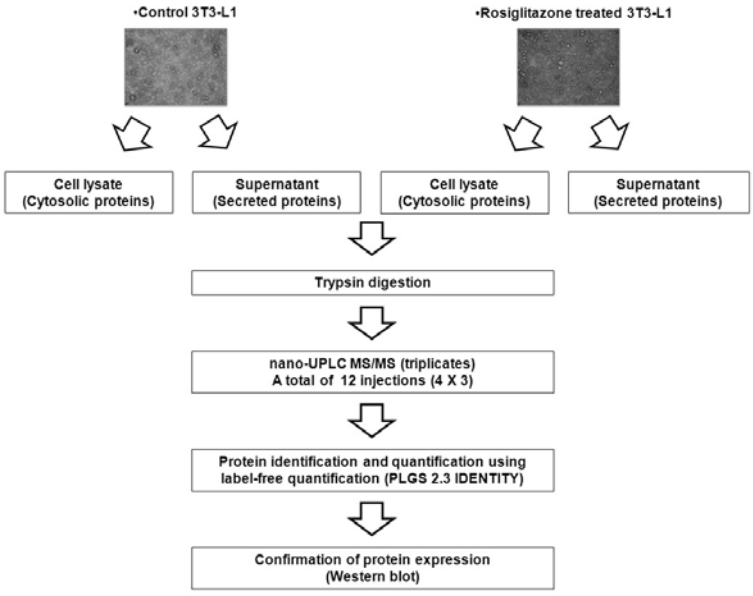



**Fig. 1.** An overview of the procedure used to identify target proteins of rosiglitazone.

4.1™. The resulting data-set was processed using Waters Protein Expression System Informatics incorporated in ProteinLynx Global SERVER (PLGS) 2.3 IDENTITY and cluster of exact mass and retention time (EMRT) (Hughes et al., 2006; Silva et al., 2006b). Briefly, spectra were collected by alternating between low- and high-collision energies with no selective mass filtering. All fragmentation data were collected for every precursor ion and were not limited by the number of MS/MS scans. In order to maximize quantitative data, intensity of each precursor peptide was collected across its entire peak. Using computational methods, fragments in the high energy scans were assigned to precursor ions based on elution profiles.

Protein identifications were assigned against an IPI_mouse database [version 3.35 containing the yeast enolase fasta sequence (swiss-prot P00924)] using precursor and fragmentation data afforded by the $MS^E$ acquisition method. Search parameter values for each precursor and associated fragment ion were set by the software using the measured mass error and intensity error obtained from processing of the raw continuum data. Mass error tolerance values were typically under 3 ppm. Peptide identifications were restricted to tryptic peptides with no more than one missed cleavage and cysteine carbamidomethylation, acetylation of N terminal, deamination of N and Q and oxidation M were set. Minimum fragment ion matches per peptide was set at 3 and minimum fragment ion matches per protein was set at 4. False positive rate was set at < 4%. Functional categorization of proteins was carried out according to gene ontology (GO) using The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003).

**Western blotting**

In order to confirm the results of LC-MS/MS quantitation, cell lysates (10 ug) was separated on 12% SDS-PAGE, and transferred to a nitrocellulose membrane (Whatman, Germany). The membrane was incubated overnight at 4°C with each antibody, including a monoclonal anti-fatty acid-binding protein, anti-adiponectin, anti-adenomatosis polyposis coli 2, anti-eukaryotic translation initiation factor 5A-1, and anti-actin antibody (Abcam Inc., USA), and then incubated with each corresponding secondary antibody, an anti-mouse IgG antibody (Cell Signaling Technology, Inc., USA) or an anti-rabbit IgG antibody (USA) for 1 h at room temperature. ECL detection reagent (Amersham Biosciences, UK) was used for detection of immunoreactive proteins.

**Measurement of transcripts by RT-PCR analysis**

Fully differentiated 3T3-L1 was treated by rosiglitazone (or pioglitazone) for short times, 6 h or 12 h. Total RNA was isolated from each 3T3-L1 adipocytes using TRIzol (Invitrogen) according to the manufacture's protocol. cDNAs were prepared by reverse transcription of 1 ug total RNA using oligo$(dT)_{15}$ and Superscript II reverse transcriptase. cDNA was amplified by 32 cycles of PCR using PCR premix (Bioneer, USA ) and following primers; for eIF5A: sense 5′-CCA TCC AAT CGG TAG TAG CG-3′; antisense, 5′- GTA ACC CGT TGA ACC CCA TT-3′; for APC2: sense 5′-5-ACC AGG ACA AAA ACC CAA TG-3′, antisense, 5′-TTC ATT GCA TGC CTA TGC TC-3′; and for GAPDH: sense 5′-TCC ACT CAC GGC AAA TTC AAC G-3′, antisense, 5′-TAG ACT CCA CGA CAT ACT CAG C-3′.

## RESULTS AND DISCUSSION

**Rosiglitazone treatment**

The flowchart in regards to the procedure used to identify target proteins of rosigiltazone is illustrated in Fig. 1. In this experiment, we attempted to view the actions of rosiglitazone on cytosolic and secreted proteins. The 3T3-L1 cell line, which consists of mouse embryo fibroblasts, was used for this experiment. To identify proteins involved in rosiglitazone action during differentiation, rosiglitazone was continuously treated after differentiation induction, and both cytosolic and secreted proteins (secretome) were identifed by nanoUPLC combin with QTOF premier. Protein identification and quantification were performed by PLGS 2.3 IDENTITY based on stringent proteomic criteria. We found a number of proteins that were expressed differentially by a label free quantification method from proteomic data and these proteins were analyzed through bioinformatic tools, such as the DAVID program, in order to find meaningful proteins expressed by the influence of rosiglitazone.

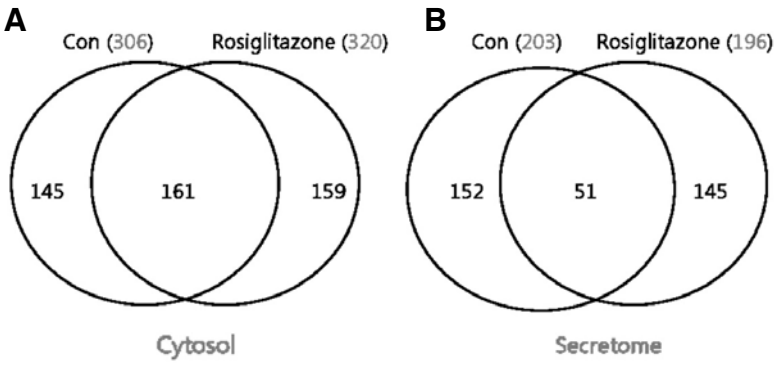



**Fig. 2.** Venn diagram of overlapping indicates the number of proteins between the two groups, control and rosiglitazone-treated. (A) In cytosolic fraction, a total of 306 proteins were identified in the control group, and 320 in the rosiglitazone-treated group. (B) In secretome, a total of 203 proteins were identified in the control group and 196 in the rosiglitazone-treated group.

**Nano-UPLC MS/MS analysis of cytosolic and secreted proteins**

Cell cytosolic and secreted proteins (secretome) were prepared from two groups (control and rosiglitazone-treated). These proteomes were subjected to tryptic digestion and tryptic peptides (1 μg from each sample) from 4 samples (control group; lysate and secretome, rosiglitazone-treated group; lysate and secretome) were analyzed by nano-UPLC MS/MS using the $MS^E$ method. For analytical replicate, each sample was analyzed three times, and, finally, 12 injections for MS/MS analysis were processed for this study. MS/MS raw data were processed and searched against IPI rat ver. 3.65 using PLGS 2.3. Details on protein identification are described under "Materials and Methods", and also according to general procedures that have been previously reported (Silva et al., 2006a; Vissers et al., 2007). Figure 2 shows the overlap of protein identifications between the two groups (control and rosiglitazone-treated); from the cytosolic fraction, a total of 306 proteins were identified in the control group, and 320 in the rosiglitazone-treated group (Fig. 2A). In addition, from secretome analysis, a total of 203 proteins were identified in the control group and 196 in the rosiglitazone-treated group (Fig. 2B). Finally, a total of 646 non-redundant proteins were identified, based on analysis of cytosol (465) and secretome (348) of the two groups.

**Proteins differentially expressed by rosiglitazone in adipocytes**

For quantification and statistical analysis, proteins were quantified from at least two of three MS/MS data sets per one condition sample. LC-MS/MS was used in the conduct of quantitative analysis between normal and rosiglitazone-treated proteomes. Through label-free quantification after LC-MS/MS, we were able to identify rosiglitazone-induced proteins. In fact, label free quantification technology is a very advanced and high-throughput proteomic method featuring a high performance mass apparatus and powerful analysis software based on analysis of exact mass retention time (EMRT) of each peptide from samples. Furthermore, the technology enables rapid identification of large numbers of proteins in a relatively short time (Wong et al., 2008).

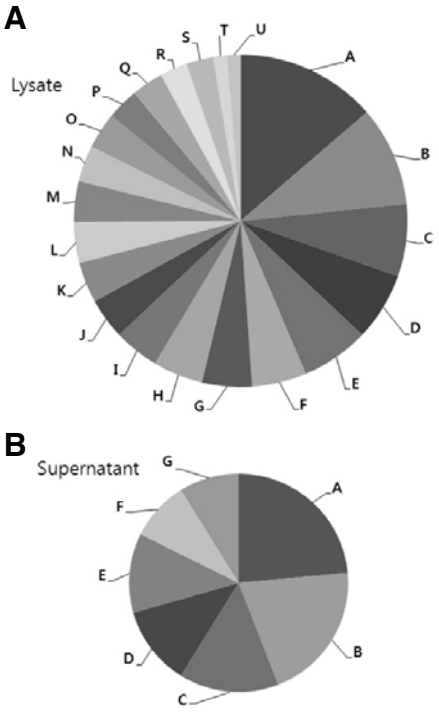
For analysis of 3T3-L1 cell lysates, a total of 465 proteins were identified. A total of 218 proteins were found to differ consistently in expression level between the two groups (116 were upregulated (≥ 1.5 fold change and unique) and 102 were down regulated (≤ 0.67 fold change and unique). Second, for secretome analysis, a total 348 of proteins were identified. A total of 116 proteins were found to be differentially expressed (≥ 1.5 fold change; 56, ≤ 0.67 fold change; 60).

For analysis of the function of proteins identified from 3T3-L1 cells, differentially expressed proteins (DEPs) from each proteome were analyzed using the DAVID GO tool (DAVID Bioinformatics Resources 6.7, http://david.abcc.ncifcrf.gov/ summary.jsp), as shown at Fig. 3. For the lysate proteome, various proteins showed differential expression related to the following functions: oxidation-reduction, generation of precursor metabolites and energy, protein localization, fatty acid metabolic processes, glucose metabolic processes, fat cell differentiation, and so on. For the secretome, the following functions were shown: macromolecular complex assembly, protein localization, defense response, generation of precursor metabolites and energy, glucose metabolic processes, and fat cell differentiation. Among these, we have presented a partial list of proteins showing differential expression with biological significance in Table 1.

As expected, the many proteins that are known to be related to the PPAR signaling pathway include fatty acid translocase (FAT)/CD36 (fatty acid binding), fatty acid binding proteins (fatty acid transport), Lipoprotein lipase (fatty acid transport), acetyl CoA acyltransferase (fatty acid oxidation), carnitine palmitoyltransferase 2 (fatty acid oxidation), sterol carrier protein (fatty acid oxidation), adiponectin (adipocyte differentiation, insulin sensitivity), ubiquitin C (ubiquitination), and phosphoenolpyruvate carboxykinase (gluconeogenesis) (Table 1).

PPARγ plays an important role in regulating lipid partitioning in adipocytes. TZDs are known to activate gene expression related to an increase in lipid transportation into adipocytes. Major target genes involved in this pathway include lipoprotein lipase (Schoonjans et al., 1996), fatty acid translocase (FAT)/CD36 (Pravenec et al., 2003), fatty-acid transport protein (Frohnert et al., 1999), and LDL receptor 1 (Chui et al., 2005), which facilitate adipocyte uptake of circulating fatty acids. In this study, increase of all of these important proteins was detected in the TZD treatment group. Lipoprotein has a function in hydrolysis of triglycerides of circulating chylomicrons and very low density lipoproteins (VLDL). Fatty acid translocase (FAT)/CD36 can bind to oxidized LDL and long chain fatty acids and may function in transport and as a regulator of fatty acid transport (Pravenec et al., 2003). Fatty acid binding protein 4 can bind both long chain fatty acids and retinoic acid and deliver long-chain fatty acids to corresponding receptors in the nucleus. Fatty acid binding protein 5 also has high specificity for fatty acid (C18 chain length).

The protein expression of acetyl CoA acyltransferase (fatty acid oxidation), carnitine palmitoyltransferase 2 (fatty acid oxidation), and sterol carrier proteins (fatty acid oxidation) are up regulated in this result. Flux of fatty acids into mitochondria is controlled by a carnitine-dependent facilitated transport system. Of particular interest, one of its critical components, carnitine palmitoyl transferase I (*cpt1*), is strongly induced by peroxisome proliferators and fatty acids (Brady et al., 1989; Foxworthy et al., 1990). On the other hand, we identified upregulation of CPT 2.




| | | |
|---|---|---|
| A | oxidation reduction | 13.70% |
| B | generation of precursor metabolites and energy | 9.70% |
| C | protein localization | 7.00% |
| D | fatty acid metabolic process | 6.60% |
| E | homeostatic process | 6.60% |
| F | cofactor metabolic process | 5.30% |
| G | translation | 4.80% |
| H | macromolecular complex assembly | 4.80% |
| I | protein complex biogenesis | 4.40% |
| J | glucose metabolic process | 4.00% |
| K | energy derivation by oxidation of organic compounds | 4.00% |
| L | cellular respiration | 4.00% |
| M | nitrogen compound biosynthetic process | 4.00% |
| N | lipid catabolic process | 3.50% |
| O | organic acid catabolic process | 3.50% |
| P | acetyl-CoA catabolic process | 3.10% |
| Q | electron transport chain | 3.10% |
| R | alcohol catabolic process | 2.60% |
| S | glycolysis | 2.60% |
| T | protein polymerization | 1.30% |
| U | fat cell differentiation | 1.30% |

| | | |
|---|---|---|
| A | homeostatic process | 23.50% |
| B | defense response | 20.60% |
| C | generation of precursor metabolites and energy | 14.70% |
| D | glucose metabolic process | 11.80% |
| E | fat cell differentiation | 11.80% |
| F | alcohol catabolic process | 8.80% |
| G | glycolysis | 8.80% |

**Fig. 3.** Functional analysis of proteins differentially expressed from each proteome. The categories of proteins were generated using the program provided by the DAVID GO tool for (A) differential expressed proteins from the cytosolic fraction and (B) differential expressed proteins from secreted fraction.

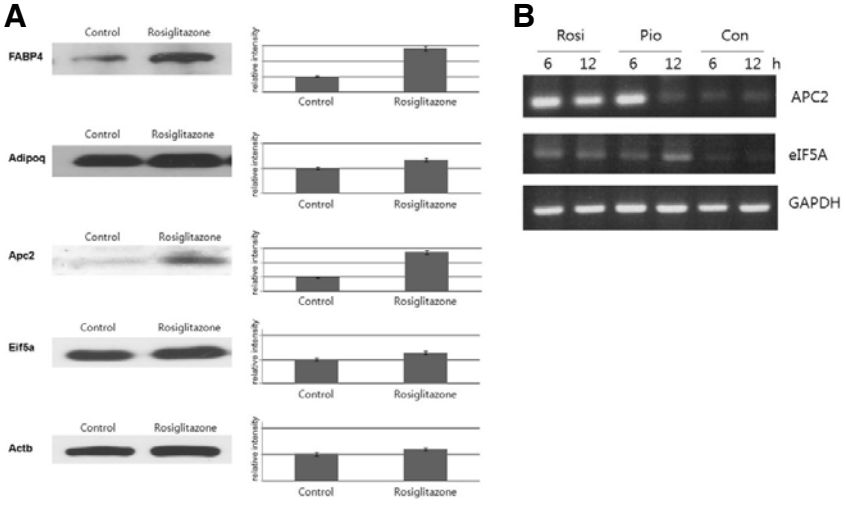



**Fig. 4.** Confirmation of MS quantification results by western blot analysis and RT-PCR. (A) To confirm MS quantification results, using the commercially available antibody, we selected certain proteins, in-cluding fatty acid binding protein (FABP4), adiponectin (Adipoq), APC2, and eIF5A. Lysate (3 μg) of 3T3-L1 adipocytes was separated by SDS-PAGE and detected by each antibody. Right panel indicates the intensities from Western blot analysis. Each statistical analysis was carried out using Student *t*-test ($P < 0.05$) from three independent experiments. (B) RT-PCR for the detection of APC2 and eIF5A mRNAs levels in differentiated 3T3-L1 adipocytes. Cells were incubated with rosiglitazone or pioglitazone for 6 h and 12 h and compared with untreated control. A representative experiment was shown.

The gene encoding *cpt 2* also probably represents another PPARγ target gene. PPARγ directly regulates mitochondrial β-oxidation by stimulating expression of the medium-chain acyl-CoA dehydrogenase (*Mcad*) gene, the promoter of which contains a functional PPRE (Gulick et al., 1994). In addition, sterol carrier protein-2 can mediate the transfer of phospholipids, cholesterol and gangliosides between membranes (Schroeder et al., 1990; van Amerongen et al., 1989).

Prior to their identification as PPARγ ligands, TZDs were found to be effective for treatment of type 2 diabetes, as they directly reduced the systemic insulin resistance of peripheral tissues (Nolan et al., 1994). In this context, we identified adiponectin as one of the up-regulated proteins in the rosiglitazone-treated group. TZDs are known to induce expression of the insulin-sensitizing factor, adiponectin, and to simultaneously reduce adipocyte expression of several insulin resistance-promoting polypeptides. This causes lowering of serum fatty-acid levels by promoting flux into adipose tissue and reducing adipocyte production of cortisol (Rangwala and Lazar, 2004). Also, the enzymes, including phosphoenolpyruvate carboxylkinase catalyzing glycolysis/gluconeogenesis (Tontonoz et al., 1995; Tordjman et al., 2003), were upregulated in this study, possibly in favor of gluconeogenesis.

TZDs may increase the number of small adipocytes; however, of particular interest, they also decrease the number of large adipocytes by inducing adipocyte apoptosis (Okuno et al., 1998; Yamauchi et al., 2001). We identified two proteins that might explain these phenomena of apoptosis and cell division in adipocytes treated by rosiglitazone.

First, Adenomatosis polyposis coli 2 (APC2) protein was up regulated in rosiglitazone-treated adipocytes. This protein helps control cell division, attachment, or movement of a cell. This

**Table 1.** Partial list of proteins showing differential expression with biological significance.

| Function | Protein name | PLGS score[a] | Fold changes of cytosolic proteins[b] | Fold changes of secreted proteins[c] |
|---|---|---|---|---|
| PPAR signaling pathway | | | | |
| | Fatty acid translocase(FAT)/CD36 | 66.66 | 1.88 ± 0.62 [0.04] | - |
| | Fatty acid-binding protein 4 | 181.32 | 2.1 ± 0.1 [0.00] | - |
| | Fatty acid-binding protein 5 | 579.19 | 1.54 ± 0.04 [0.00] | 1.73 ± 0.06 [0.00] |
| | Lipoprotein lipase | 118.85 | rosi unique | 1.65 ± 0.11 [0.00] |
| | Acetyl-Coenzyme A acyltransferase 2 | 153.57 | rosi unique | - |
| | Carnitine palmitoyltransferase 2 | 72.35 | rosi unique | - |
| | Sterol carrier protein-2 | 55.78 | rosi unique | - |
| | Adiponectin | 184.57 | 1.68 ± 0.31 [0.00] | 1.32 ± 0.08 [0.00] |
| | Ubc protein | 227.62 | 1.67 ± 0.15 [0.00] | 1.04 ± 0.09 [0.18] |
| | Phosphoenolpyruvate carboxykinase 2 | 170.46 | rosi unique | - |
| | 3-ketoacyl-CoA thiolase B | 66.79 | 3.19 ± 0.27 [0.00] | - |
| | 3-ketoacyl-CoA thiolase A | 80.54 | 2.53 ± 0.3 [0.00] | - |
| | Ubiquitin B | 161.91 | 1.67 ± 0.16 [0.00] | 1.05 ± 0.1 [0.13] |
| | Ribosomal protein S27a | 89.53 | 1.75 ± 0.19 [0.00] | 1.35 ± 0.06 [0.00] |
| | Keratinocyate lipid-binding protein | 148.44 | 2.08 ± 0.11 [0.00] | rosi unique |
| | Isoform SCP2 of Non-specific lipid-transfer protein | 67.84 | rosi unique | - |
| Other fatty acid metabolism | | | | |
| | Aldehyde dehydrogenase 9, subfamily A1 | 99.23 | rosi unique | - |
| | Hydroxyacyl-Coenzyme A dehydrogenase 3-ketoacyl-Coenzyme A thiolase enoyl-Coenzyme A hydratase (Trifunctional protein), alpha subunit | 424.39 | 1.52 ± 0.11 [0.00] | - |
| | Dodecenoyl-Coenzyme A delta isomerase | 62.88 | 1.75 ± 0.39 [0.01] | 1.23 ± 0.05 [0.00] |
| | 3-ketoacyl-CoA thiolase, mitochondrial | 153.57 | rosi unique | - |
| Biosynthesis of unsaturated fatty acids | | | | |
| | Isoform 2 of Estradiol 17-beta-dehydrogenase 12 | 107.11 | rosi unique | - |
| | Isoform 1 of Estradiol 17-beta-dehydrogenase 12 | 147.33 | rosi unique | - |
| | Isoform C of Cytosolic acyl coenzyme A thioester hydrolase | 73.84 | rosi unique | - |
| | Isoform A of Cytosolic acyl coenzyme A thioester hydrolase | 72.02 | rosi unique | - |
| | Isoform B of Cytosolic acyl coenzyme A thioester hydrolase | 80.82 | rosi unique | - |
| | Isoform D of Cytosolic acyl coenzyme A thioester hydrolase | 81.32 | rosi unique | - |
| Glycolysis / Gluconeogenesis | | | | |
| | Glyceraldehyde-3-phosphate-dehydrogenase isoform 2 | 242.1 | rosi unique | - |
| | Ldhb 21 kDa protein | 101.84 | 1.72 ± 0.24 [0.00] | - |
| | Eno3 Beta-enolase | 102.52 | 1.7 ± 0.2 [0.00] | - |
| | Dihydrolipoyl dehydrogenase | 88.4 | 2.48 ± 0.3 [0.00] | - |
| | 6-phosphofructokinase, muscle type | 45.61 | rosi unique | - |
| | Pyruvate kinase isozymes R L | 196.51 | rosi unique | - |
| Apoptosis and cell division | | | | |
| | Voltage-dependent anion-selective channel protein 2 | 90.05 | con unique | - |
| | Adenomatosis polysis coli 2 | 148.44 | 2.08 ± 0.11 [0.00] | - |
| | Eukaryotic translation initiation factor 5A-1 | 124.93 | 2.05 ± 0.41 [0.00] | rosi unique |

[a], PLGS score (corresponding to lysate proteins) calculated from Software IDENTITY.
[b,c], show the rosiglitazone/control ratio ± SD [P-value].

protein is also helpful in control of the chromosome number in cells after cell division is correct. The APC protein presents these functions through its relationship with other proteins, i.e. beta-catenin (Wnt signaling pathway). These observations might suggest that regulation of APC2 by rosiglitazone could increase the number of small adipocytes. Furthermore, *APC* is classified as a tumor suppressor gene. According to reports, TZDs promote growth in colon tumors with mutations in the APC gene

(Lefebvre et al., 1998; Saez et al., 1998; Thompson, 2007).

Second, eukaryotic translation initiation factor 5A-1 (eIF5A) was up regulated in rosiglitazone-treated adipocytes. This protein, previously known as eIF4D and IF-M2Ba, is a highly conserved 17-kDa protein that was originally known as a translation initiation factor, initiating formation of the first peptide bond in mRNA translation (Kemper et al., 1976; Saini et al., 2009). However, this protein has recently been thought to function in translation of mRNAs that encode proteins essential for G1-S transition of the cell cycle (Chatterjee et al., 2006), for cytotoxic stress responses (Rahman-Roblick et al., 2007), and for propagation of human immunodeficiency virus (Bevec et al., 1996). Therefore, eIF5A is a major factor that controls the balance between cell proliferation and death. Of particular interest, the *eIF5A* gene on the distal arm of mouse chromosome 11 is located within the type 1 diabetes susceptibility locus *Idd4.* Recently, a group identified eIF5A as a critical regulator of the inflammatory response in mouse pancreatic islets (Maier et al., 2010). Therefore, eIF5A might play a role in function related to the decrease of large adipocytes and the increase of small adipocytes.

**Confirmation of proteomic data by Western blot analysis and RP-PCR**

To confirm MS quantification results, using the commercially available antibody, we selected certain proteins, including fatty acid binding protein (FABP4), adiponectin (Adipoq), APC2, and eIF5A. As expected, FABP4 and adiponectin, which are important for fatty acid transport into adipocytes and for insulin sensitivity, respectively, were upregulated in rosiglitazone-treated adipocytes (Fig. 4A). Furthermore, two proteins, APC2 and eIF5A, were upregulated in rosiglitazone-treated adipocytes as shown MS quantification result (Fig. 4A).

To confirm whether the two proteins APC2 and eIF5A are direct targets of rosiglitazone, mRNA levels of APC2 and eIF5A were studied using fully differentiated 3T3-L1 with short rosiglitazone treatments, for 6 or 12 h. In addition, to study whether these two proteins are general targets of PPARγ ligands, pioglitazone, one of PPARγ ligands, was treated under same conditions as rosigitazone treatment. As shown at Fig. 4B, mRNAs level of APC2 and eIF5A by treatment of two drugs were significantly increased at 6 h and 12 h compared to control treatments except a decrease of APC2 mRNA at 12 h of pioglitazone treatment.

## CONCLUSION

PPARγ play many important roles in various fields including adipocyte differentiation, adipocyte metabolism, insulin sensitivity, body weight, and inflammation. In this study, we attempted to understand the action mechanism of rosiglitazone through in-depth proteomic approaches, using cytosolic and secreted proteins. From analysis of two data sets, we identified and quantified 646 proteins through label-free quantification method using nano-UPLC Q-TOF MS/MS from rosiglitazone-treated 3T3-L1 adipocytes as compared to controls. As expected, many differentially expressed proteins were related to fatty acid binding, fatty acid transport, fatty acid oxidation, adipocyte differentiation, gluconeogenesis, and so on. In addition, we identified two novel proteins, APC2 and eIF5A, which might explain the regulation of the number of small adipocytes and large adipocytes. This approach revealed the various targets of rosiglitazone at the protein level and would be helpful in our further understanding of the role of rosiglitazone in adipocytes.

## ACKNOWLEDGMENTS

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (Ministry of Education and Human Resources Department, Basic Research Promotion Fund) (KRF-2008-331-C00181) and was supported by the Grant of the Korean Ministry of Education, Science and Technology (The Regional Core Research Program / Anti-aging and Well-being Research Center).

## REFERENCES

Ahmed, M., Neville, M.J., Edelmann, M.J., Kessler, B.M., and Karpe, F. (2010). Proteomic analysis of human adipose tissue after rosiglitazone treatment shows coordinated changes to promote glucose uptake. Obesity *18*, 27-34.

American Diabetes Association. (2008). Economic costs of diabetes in the U.S. in 2007. Diabetes Care *31*, 596-615.

Berger, J., and Moller, D.E. (2002). The mechanisms of action of PPARs. Annu. Rev. Med. *53*, 409-435.

Bevec, D., Jaksche, H., Oft, M., Wohl, T., Himmelspach, M., Pacher, A., Schebesta, M., Koettnitz, K., Dobrovnik, M., Csonga, R., et al. (1996). Inhibition of HIV-1 replication in lymphocytes by mutants of the Rev cofactor eIF-5A. Science *271*, 1858-1860.

Boden, G., Cheung, P., Mozzoli, M., and Fried, S.K. (2003). Effect of thiazolidinediones on glucose and fatty acid metabolism in patients with type 2 diabetes. Metabolism *52*, 753-759.

Brady, P.S., Marine, K.A., Brady, L.J., and Ramsay, R.R. (1989). Co-ordinate induction of hepatic mitochondrial and peroxisomal carnitine acyltransferase synthesis by diet and drugs. Biochem. J. *260*, 93-100.

Buschemeyer, W.C., 3rd, and Freedland, S.J. (2007). Obesity and prostate cancer: epidemiology and clinical implications. Eur. Urol. *52*, 331-343.

Castelein, H., Gulick, T., Declercq, P.E., Mannaerts, G.P., Moore, D.D., and Baes, M.I. (1994). The peroxisome proliferator activated receptor regulates malic enzyme gene expression. J. Biol. Chem. *269*, 26754-26758.

Chatterjee, I., Gross, S.R., Kinzy, T.G., and Chen, K.Y. (2006). Rapid depletion of mutant eukaryotic initiation factor 5A at restrictive temperature reveals connections to actin cytoskeleton and cell cycle progression. Mol. Genet. Genomics *275*, 264-276.

Chui, P.C., Guan, H.P., Lehrke, M., and Lazar, M.A. (2005). PPAR-gamma regulates adipocyte cholesterol metabolism via oxidized LDL receptor 1. J. Clin. Invest. *115*, 2244-2256.

Cook, K.S., Min, H.Y., Johnson, D., Chaplinsky, R.J., Flier, J.S., Hunt, C.R., and Spiegelman, B.M. (1987). Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve. Science *237*, 402-405.

de Souza, C.J., Eckhardt, M., Gagen, K., Dong, M., Chen, W., Laurent, D., and Burkey, B.F. (2001). Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. Diabetes *50*, 1863-1871.

Dennis, G., Jr., Sherman, B.T., Hosack, D.A., Yang, J., Gao, W., Lane, H.C., and Lempicki, R.A. (2003). DAVID: database for annotation, visualization, and integrated discovery. Genome Biol. *4*, P3.

Ebrahimpour, P., Fakhrzadeh, H., Pourebrahim, R., Hamidi, A., and Larijani, B. (2006). Metabolic syndrome and related insulin levels in obese children. Metab. Syndr. Relat. Disord. *4*, 172-178.

Flier, J.S., Cook, K.S., Usher, P., and Spiegelman, B.M. (1987). Severely impaired adipsin expression in genetic and acquired obesity. Science *237*, 405-408.

Fonseca, V. (2003). Effect of thiazolidinediones on body weight in patients with diabetes mellitus. Am. J. Med. *115*, 42S-48S.

Foxworthy, P.S., Perry, D.N., Hoover, D.M., and Eacho, P.I. (1990). Changes in hepatic lipid metabolism associated with lipid accumulation and its reversal in rats given the peroxisome proliferator LY171883. Toxicol. Appl. Pharmacol. *106*, 375-383.

Frohnert, B.I., Hui, T.Y., and Bernlohr, D.A. (1999). Identification of a functional peroxisome proliferator-responsive element in the murine fatty acid transport protein gene. J. Biol. Chem. *274*, 3970-3977.

Green, H., and Kehinde, O. (1974). Sublines of mouse 3T3 cells that accumulate lipid. Cell *1*, 113-116.

Gregoire, F.M., Smas, C.M., and Sul, H.S. (1998). Understanding adipocyte differentiation. Physiol. Rev. *78*, 783-809.

Gulick, T., Cresci, S., Caira, T., Moore, D.D., and Kelly, D.P. (1994). The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. Proc. Natl. Acad. Sci. USA *91*, 11012-11016.

Halberg, N., Wernstedt-Asterholm, I., and Scherer, P.E. (2008). The adipocyte as an endocrine cell. Endocrinol. Metab. Clin. North Am. *37*, 753-768, x-xi.

Hughes, M.A., Silva, J.C., Geromanos, S.J., and Townsend, C.A. (2006). Quantitative proteomic analysis of drug-induced changes in mycobacteria. J. Proteome Res. *5*, 54-63.

Kemper, W.M., Berry, K.W., and Merrick, W.C. (1976). Purification and properties of rabbit reticulocyte protein synthesis initiation factors M2Balpha and M2Bbeta. J. Biol. Chem. *251*, 5551-5557.

Kim, K.H., Lee, K., Moon, Y.S., and Sul, H.S. (2001). A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. J. Biol. Chem. *276*, 11252-11256.

Lefebvre, A.M., Chen, I., Desreumaux, P., Najib, J., Fruchart, J.C., Geboes, K., Briggs, M., Heyman, R., and Auwerx, J. (1998). Activation of the peroxisome proliferator-activated receptor gamma promotes the development of colon tumors in C57BL/6J-APCMin/+ mice. Nat. Med. *4*, 1053-1057.

Maier, B., Ogihara, T., Trace, A.P., Tersey, S.A., Robbins, R.D., Chakrabarti, S.K., Nunemaker, C.S., Stull, N.D., Taylor, C.A., Thompson, J.E., et al. (2010). The unique hypusine modification of eIF5A promotes islet beta cell inflammation and dysfunction in mice. J. Clin. Invest. *120*, 2156-2170.

Nolan, J.J., Ludvik, B., Beerdsen, P., Joyce, M., and Olefsky, J. (1994). Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. N. Engl. J. Med. *331*, 1188-1193.

Okuno, A., Tamemoto, H., Tobe, K., Ueki, K., Mori, Y., Iwamoto, K., Umesono, K., Akanuma, Y., Fujiwara, T., Horikoshi, H., et al. (1998). Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. J. Clin. Invest. *101*, 1354-1361.

Pravenec, M., Landa, V., Zidek, V., Musilova, A., Kazdova, L., Qi, N., Wang, J., St Lezin, E., and Kurtz, T.W. (2003). Transgenic expression of CD36 in the spontaneously hypertensive rat is associated with amelioration of metabolic disturbances but has no effect on hypertension. Physiol. Res. *52*, 681-688.

Rahman-Roblick, R., Roblick, U.J., Hellman, U., Conrotto, P., Liu, T., Becker, S., Hirschberg, D., Jornvall, H., Auer, G., and Wiman, K.G. (2007). p53 targets identified by protein expression profiling. Proc. Natl. Acad. Sci. USA *104*, 5401-5406.

Rajala, M.W., Obici, S., Scherer, P.E., and Rossetti, L. (2003). Adipose-derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production. J. Clin. Invest. *111*, 225-230.

Rangwala, S.M., and Lazar, M.A. (2004). Peroxisome proliferator-activated receptor gamma in diabetes and metabolism. Trends Pharmacol. Sci. *25*, 331-336.

Saez, E., Tontonoz, P., Nelson, M.C., Alvarez, J.G., Ming, U.T., Baird, S.M., Thomazy, V.A., and Evans, R.M. (1998). Activators of the nuclear receptor PPARgamma enhance colon polyp formation. Nat. Med. *4*, 1058-1061.

Saini, P., Eyler, D.E., Green, R., and Dever, T.E. (2009). Hypusine-containing protein eIF5A promotes translation elongation. Nature *459*, 118-121.

Schoonjans, K., Watanabe, M., Suzuki, H., Mahfoudi, A., Krey, G., Wahli, W., Grimaldi, P., Staels, B., Yamamoto, T., and Auwerx, J. (1995). Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter. J. Biol. Chem. *270*, 19269-19276.

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A.M., Heyman, R.A., Briggs, M., Deeb, S., Staels, B., and Auwerx, J. (1996). PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. EMBO J. *15*, 5336-5348.

Schroeder, F., Butko, P., Hapala, I., and Scallen, T.J. (1990). Intermembrane cholesterol transfer: role of sterol carrier proteins and phosphatidylserine. Lipids *25*, 669-674.

Silva, J.C., Denny, R., Dorschel, C., Gorenstein, M.V., Li, G.Z., Richardson, K., Wall, D., and Geromanos, S.J. (2006a). Simultaneous qualitative and quantitative analysis of the Escherichia coli proteome: a sweet tale. Mol. Cell. Proteomics *5*, 589-607.

Silva, J.C., Gorenstein, M.V., Li, G.Z., Vissers, J.P., and Geromanos, S.J. (2006b). Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. Mol. Cell. Proteomics *5*, 144-156.

Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Banerjee, R.R., Wright, C.M., Patel, H.R., Ahima, R.S., and Lazar, M.A. (2001). The hormone resistin links obesity to diabetes. Nature *409*, 307-312.

Thompson, E.A. (2007). PPARgamma physiology and pathology in gastrointestinal epithelial cells. Mol. Cells *24*, 167-176.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., and Spiegelman, B.M. (1994a). mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. *8*, 1224-1234.

Tontonoz, P., Hu, E., and Spiegelman, B.M. (1994b). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell *79*, 1147-1156.

Tontonoz, P., Hu, E., Devine, J., Beale, E.G., and Spiegelman, B.M. (1995). PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. Mol. Cell. Biol. *15*, 351-357.

Tordjman, J., Chauvet, G., Quette, J., Beale, E.G., Forest, C., and Antoine, B. (2003). Thiazolidinediones block fatty acid release by inducing glyceroneogenesis in fat cells. J. Biol. Chem. *278*, 18785-18790.

van Amerongen, A., Demel, R.A., Westerman, J., and Wirtz, K.W. (1989). Transfer of cholesterol and oxysterol derivatives by the nonspecific lipid transfer protein (sterol carrier protein 2): a study on its mode of action. Biochim. Biophys. Acta *1004*, 36-43.

Vissers, J.P., Langridge, J.I., and Aerts, J.M. (2007). Analysis and quantification of diagnostic serum markers and protein signatures for Gaucher disease. Mol. Cell. Proteomics *6*, 755-766.

Wang, P., Renes, J., Bouwman, F., Bunschoten, A., Mariman, E., and Keijer, J. (2007). Absence of an adipogenic effect of rosiglitazone on mature 3T3-L1 adipocytes: increase of lipid catabolism and reduction of adipokine expression. Diabetologia *50*, 654-665.

Wong, J.W., Sullivan, M.J., and Cagney, G. (2008). Computational methods for the comparative quantification of proteins in label-free LCn-MS experiments. Brief Bioinform. *9*, 156-165.

Yamauchi, T., Kamon, J., Waki, H., Murakami, K., Motojima, K., Komeda, K., Ide, T., Kubota, N., Terauchi, Y., Tobe, K., et al. (2001). The mechanisms by which both heterozygous peroxisome proliferator-activated receptor gamma (PPARgamma) deficiency and PPARgamma agonist improve insulin resistance. J. Biol. Chem. *276*, 41245-41254.

Yki-Jarvinen, H. (1994). Pathogenesis of non-insulin-dependent diabetes mellitus. Lancet *343*, 91-95.

Yki-Jarvinen, H. (2004). Thiazolidinediones. N. Engl. J. Med. *351*, 1106-1118.