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Identification of genes that are regulated by peroxisome proliferator activated receptor (PPAR)γ activator, troglitazone during adipocyte differentiation

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ABSTRACT

Many of the molecular details of adipogenesis, adipocyte differentiation are now known. However, a subset of genes are not fully defined whose expression changes during the adipogenesis activated by peroxisome proliferator-activated receptor (PPAR)γ. We used DNA microarrays to assess the changes in gene expression associated with adipogenesis and obesity. Mouse (*Mus musculus*) fibroblast stem cells named 3T3-L1 were treated with dexamethason and insulin to differentiate into adipocytes, then with PPARγ activator troglitazone to make an *in vitro* model for analyzing differential gene expression during adipogenesis. Total RNA was isolated from preadipocytes, troglitazone-treated and -untreated adipocytes respectively and labeled copy RNA (cRNA) was prepared through reverse transcription and *in vitro* transcription. The AB 1700 full genome expression mouse microarray which contained 37844 mouse gene oligonucleotides was hybridized with the labeled cRNAs and signals were detected. Analysis of the detected images provided identification of 27 genes which were regulated by PPARγ during adipogenesis.

Key words : adipogenesis, adipocyte, microarray, PPARγ, obesity

Introduction

Recent research shows there was a sharp increase of obesity and diabetes in USA (1). In Korea also, due to changed habits of eating and their life style, obesity and diabetes was increasing dramatically (2). Obesity is characterized by increased adipose tissue mass and the number of adipocytes is determined by the adipocyte differentiation process, adipogenesis. Peroxisome proliferator activated receptor (PPAR)γ has been known as the ultimate key regulator of adipogenesis and promotes the differentiation of preadipocytes into adipocytes. (3,4). Intense research has showed evidence that PPARγ is the target for antidiabetic drugs. The level of PPARγ 2 expression correlates with the degree of lipid accumulation (5). Although many of the molecular details of adipogenesis are now known, a subset of genes are not fully defined whose expression changes during adipogenesis activated by PPARγ. PPARγ can be activated by certain ligands and modulate the expression of its target genes. Therefore, the identification of genes regulated by PPARγ may aid in further deciphering the role of PPARγ *in vivo*. In addition, the identification of new PPARγ targets may help us use them as molecular markers to assess the antiobesity effects of pharmaceuticals *in vivo* as well as *in vitro*. We used DNA microarrays to characterize the changes in gene expression associated with adipogenesis and obesity in our model system, adipocytes. We show here that PPARγ influences the expression of numerous genes involved in lipid binding, transport and metabolism.

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Materials and Methods

3T3-L1 cell differentiation and drug treatment

The 3T3-L1 cells grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS were induced to differentiate by culturing them in induction medium (0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethason, 10 ㎍/㎖, insulin) for two days. These were then cultured in culture medium only including insulin (10% FBS, DMEM, 10 ㎍/㎖ insulin) for two days before being cultured in culture medium (10% FBS, DMEM) for 3 days in order to obtain adipocytes (6-8). The differentiation status of the cells was ascertained by in situ staining with oil-red-O (1-8-[4-(dimethylphenylazo) dimethylphenylazo]-2-naphthalenol) (9). The adipocytes were then treated with troglitazone (10 μM) for 24 hours in order to obtain adipogenesis up-regulated adipocytes.

Isolation of total RNA and reverse transcription labeling

Total RNA was prepared from preadipocytes, troglitazone-treated and-untreated adipocytes using TRIZOL® reagent (Invitrogen) and its quality was checked using Bioanalyzer 2100 (Agilent). cDNA was synthesized from total RNA through reverse transcription using T7-polydT primer. Double stranded cDNA was treated with T7 RNA polymerase to synthesize DIG-labeled copy RNA (cRNA) according to the manufacturer's manual for the reverse transcription and in vitro transcription (RT-IVT) kit (Applied Biosystems), and then labeled cRNA was cleaved into fragments.

Microarray and Hybridization

The AB 1700 full genome expression mouse microarrays (Applied Biosystems) which contained a total of 37844 mouse gene oligonucleotides were hybridized to the labeled cRNA preparations after washing to remove non-specific binding. Anti-digoxigenin-alkaline phosphatase antibody was added to the microarrays.

Chemiluminescent reaction and detection

Enhancing solution was added to the microarrays and chemiluminescent reaction was triggered with the addition of substrate. Signals were detected using the AB 1700 chemiluminescent microarray analyzer (Applied Biosystems). The detected images were analyzed using an analytical program called Avadis 3.3 (Strandgenomics).

Results and Discussion

The images of microarrays were detected by the AB 1700 chemiluminescent microarray analyzer using cRNAs prepared from preadipocytes, adipocytes and troglitazone-treated adipocytes and detected images were analyzed with Avadis 3.3. Signal intensities of hybridization controls on the microarray for each cRNA were found to be over threshold (**Table1**). Average normalized signal for each cRNA was also over threshold, indicating that the microarrays operated properly.

Based on the flag values, control genes, genes with sig-

Table 1. Microarray and hybridization control

	Preadipocyte	Adipocyte	Troglitazone -treated adipocyte	Threshold
Average normalized signal	24208.0	22161.9	23066.9	>5000.0
Average normalized S/N*	14.2	15.8	15.1	>3.0
Median assay background S/N*	0.0	0.0	0.0	<3
Median background	205.0	183.8	207.1	<600
Hybridization control 1 Cp	66871.7	73590.1	73729.4	>30000
Hybridization control 2 Cp	116499.2	130730.2	118631.7	>60000
Hybridization control 3 Cp	140363.2	155683.3	156304.5	>80000

S/N*: signal-to-noise values
Hybridization control : hybridization control between DIG-labeled cRNAs and oligonucleotides on microarrays

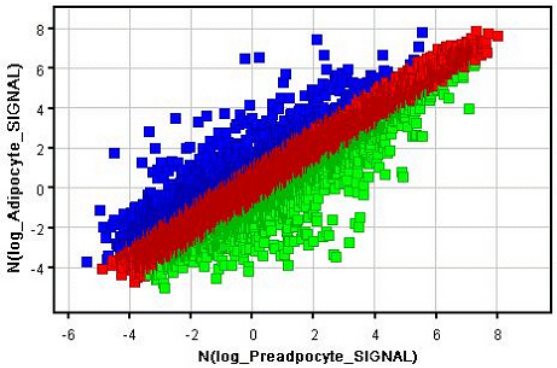

Fig 1. Scatter plot of signals detected by cRNAs prepared from preadipocytes and adipocytes. Signals were detected using the AB 1700 chemiluminescent microarray analyzer and normalized. Normalized (log preadipocyte signal); normalized (log mature adipocyte signal); Blue, red and green colors indicate over 2.0 fold, between 2.0 fold and -2.0 fold, under -2.0 fold respectively.

nal/noise of less than 3 and genes with flag value of larger than 64 were filtered out and finally 11248 genes out of 37844 mouse gene oligonucleotides were analyzed (**Table 2**). Analysis of the scatter plot of the normalized signals obtained from microarrays provided identification of 1257 genes whose signals showed 2 times more intense with cRNAs prepared from adipocytes than preadipocytes (**Fig 1**). Further characterization of these identified genes might provide more evidence to show changes in their expression during differentiation of preadipocytes into adipocytes. Five hundred genes out of them turned out to be null genes.

The PANTHER (Protein Analysis Through Evolutionary Relationships) classification system (https://pather.appilied biosystems.com) was used to classify the 1257 genes identified from our high-throughput analysis and 330 genes were classified into 78 metabolically distinct pathways (**Fig 2**). We obtained 2 times more of occurrence from the thirteen

Table 2. Flag value and meaning

Flag values	Assigned
0	measurement with no known errors or issues
1	Probe has S/N<1 and signal is replace with 1 SDEV upper limit
2	Feature centroid is from interpolated Grid position
4	Feature uses scaled pixels from CLs image
8	Partial saturation of feature CL quant pixels(0.5[P.maxsatfrac])
16	Partial saturation of feature quant pixels(0.5[P.maxsatfrac])
32	Partial saturation of pixels (1 or more) in local BG annulus.
64	Feature has pixels rejected in fit
128	Null
256	Null
512	Null
1024	Feature is outside optimal position limits
2048	Feature has FL neighbor with quantification problem
4096	Feature has CL neighbor with quantification problem
8192	Feature has poor correlation(no chosen threshold yet)
16384	Feature has low S/N(<5 of threshold)
32768	Feature has poor fit (QCmetric2<0.66)
65536	Null
131072	Null
262144	Null
524588	Null
1048576	Quantification returned a NaN value
2097152	All pixels in local BG saturated
4194304	Full saturation of feature quant pixels(>half[P.maxsatfrac])
8388608	Full saturation of feature CL feature CL quant pixels(>half[P.maxsatfrac])
16777216	Feature is too close to edge of image for quantification
33554432	Feature has no Grid position

Multiple flags are additive. Flags=1028 equals 1024 + 4 + 0 and decodes as "has replace pixels form CLs image", "outside optimal quantification limits" and "detected".
Flags 1~64 are informational.
Flags 1024~32768 are quality issues.
Flags 1048576~16777216 are failures in quantification.

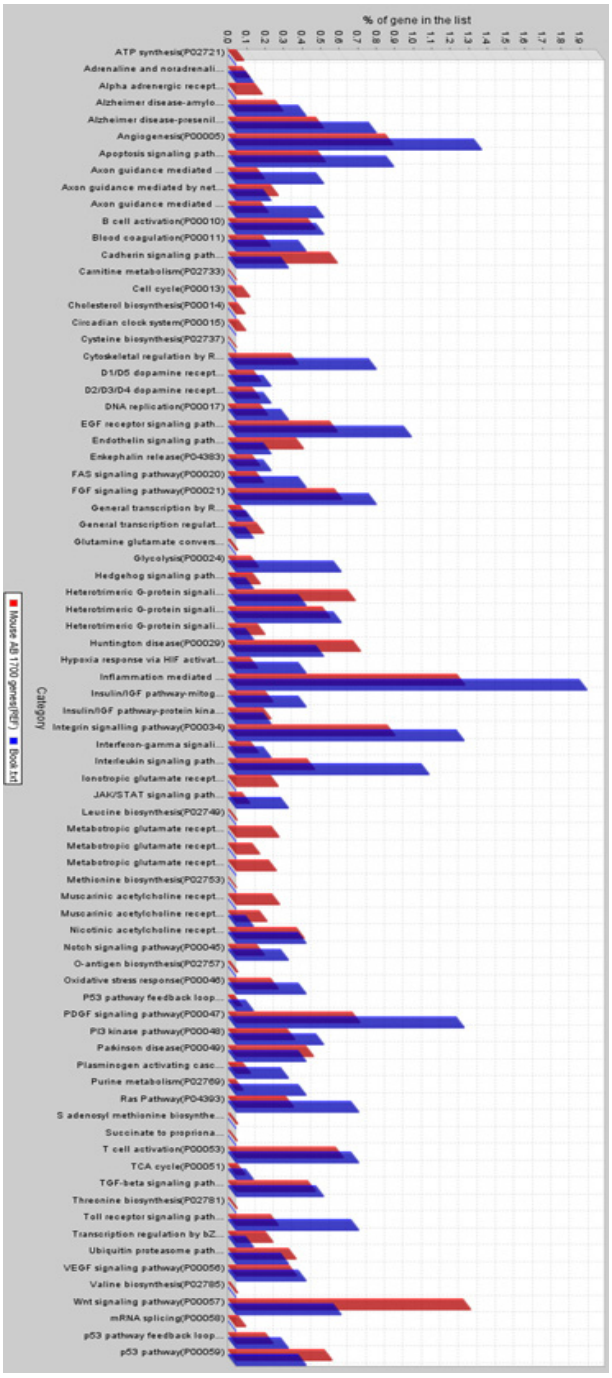

Fig 2. Occurrence of the genes in the pathway classified by the PANTHER (Protein Analysis Through Evolutionary Relationships) system. The 375 genes identified from high-throughput analysis were classified into 78 metabolically distinct pathways and percentage of gene occurrence was compared between genes on the microarray itself (red) and differentially expressed genes (blue) from adipocytes.

groups out of 78, which include glycolysis, JAK-STAT signaling pathway and purine metabolism. The identified 1257 genes were also classified in term of biological process and we could match 829 genes with known biological processes while 428 genes were not defined (**Fig 3**). The identified processes include JAK-STAT cascade and lipid and fatty acid transporter. Especially, genes which were related to the biological processes involved in lipid binding, transport or metabolism were found to be more prevalent according to the percentage of occurrence. These genes showing higher occurrence in the adipocytes compared with preadipocytes might suggest that these may be involved in adipogenesis process.

Analysis of the scatter plot of the normalized signals obtained from adipocytes with or without troglitazone treatment provided identification of 38 genes out of 1257 genes whose signals showed 2 times more increase or decrease in intensity with cRNAs prepared from troglitazone-treated adipocytes than untreated adipocytes (**Fig 4**). Analysis of the detected images provided identification of 27 genes which were regulated by PPARγ during adipogenesis (**Table 3**). Fourteen genes out of them were found to be up-regulated by drug treatment while 24 genes were down-regulated. The differentially expressed 38 genes include 11 unknown genes.

The PPARγ has been known to play a key role in adipocyte gene expression and differentiation. The adipogenic effects of PPARγ activation appear to perform through the transactivation of adipocyte gene promoters (9-16). In our system, twenty seven genes were identified to be regulated by PPARγ during adipogenesis. Among them, 12 genes were up-regulated by PPARγ of which 3 genes appear to be involved in lipid transport and metabolism, while 15 genes were down-regulated of which 4 genes were related to cell growth, cell migration and transcription.

In conclusion, we have demonstrated a differential gene expression profile of a subset of genes in adipocytes compared to preadipocytes and also in adipocytes activated by PPARγ relative to nontreated ones. These results suggest that further study to dissect the PPARγ target genes will provide a basis to develop molecular markers for obesity and type 2 diabetes.

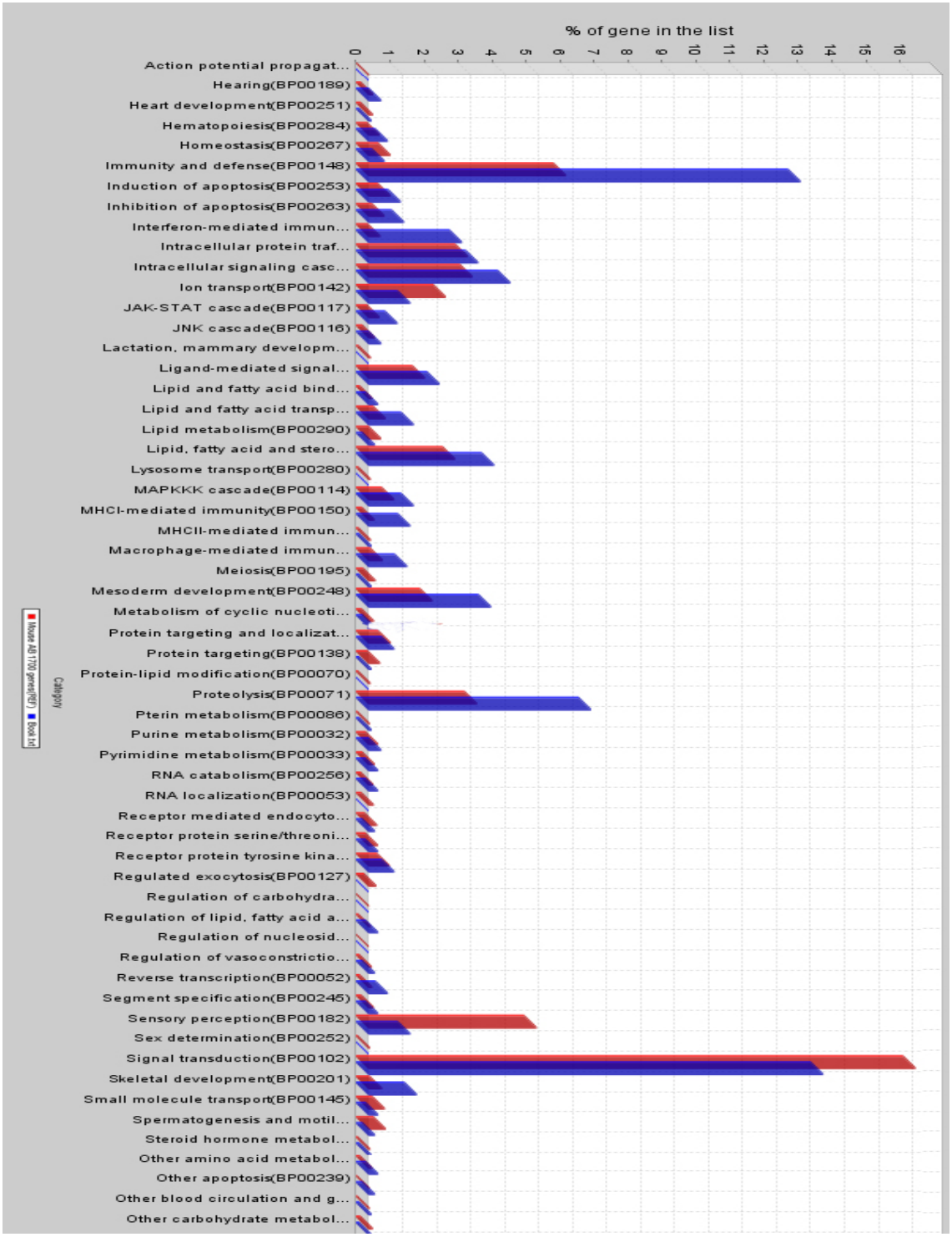

Fig 3. Occurrence of the genes in the biological process classified by the PANTHER (Protein Analysis Through Evolutionary Relationships) system. The 858 genes identified from high-throughput analysis were classified into distinct biological processes and percentage of gene occurrence was compared between genes on the microarray itself (red) and differentially expressed genes (blue) from adipocytes.

Table 3. Identification of genes that are regulated during adipocyte differentiation and activated by peroxisome proliferator activated receptor (PPAR)γ activator, troglitazone during adipogenesis

Gene Name	PANCHER GENE ID	Mature / pre adipocyte fold	Troglitazone / mature adipocyte fold	GO Biological Process
RIKEN cDNA 1500001H12 gene	mCG23290.2	3.5	-2.7	unknown
RIKEN cDNA 2510004L01 gene	mCG17799.1	36.8	-2.5	unknown
abhydrolase domain containing 3	mCG14675.2	2.8	2.0	unknown
zinc finger protein 336	mCG18946.2	3.9	-2.9	transcription
connective tissue growth factor	mCG6745.2	2.7	-2.2	Cell growth
RIKEN cDNA 9530081K03 gene	mCG7096.2	3.0	2.0	Cell growth
cytochrome P450, family 2, subfamily f, polypeptide 2	mCG22749.2	3.2	2.2	electron transport
RIKEN cDNA A630035I11 gene	mCG140154.1	2.8	-2.3	transcription
DnaJ (Hsp40) homolog, subfamily B, member 4	mCG21837.2	2.1	-2.0	protein folding
acyl-CoA synthetase long-chain family member 1	mCG1844.2	2.0	2.4	fatty acid metabolism
stearoyl-Coenzyme A desaturase 1	mCG131749.1	5.4	-3.4	fatty acid metabolism
single WAP motif protein 2	mCG5443.1	2.9	-2.4	xenobiotic metabolism
fatty acid binding protein 4, adipocyte	mCG1645.2	6.5	3.9	transport
fatty acid binding protein 4, adipocyte	mCG1645.2	4.2	6.7	transport
solute carrier family 2 (facilitated glucose transporter), member 1	mCG6287.1	4.8	-2.6	transport
sortilin 1	mCG4048.2	12.0	2.2	endocytosis
interleukin 6	mCG11634.2	7.7	-3.3	immune response
phosphodiesterase 1B, Ca2+-calmodulin dependent	mCG15675.3	2.5	2.6	signal transduction
exostoses (multiple) 2	mCG18032.3	2.1	-2.4	cell growth
AMP deaminase 3	mCG7237.2	6.3	-2.7	nucleotide metabolism
epoxide hydrolase 2, cytoplasmic	mCG2504.2	3.8	3.8	xenobiotic metabolism
DNA segment, Chr 7, ERATO Doi 458, expressed;poliovirus receptor	mCG4885.2	3.3	-2.4	cell migration
tenascin C	mCG20077.2	2.0	-2.3	unknown
polydomain protein	mCG12393.1	4.0	-2.0	unknown
claudin 15	mCG18027.1	3.4	2.0	unknown
oxidized low density lipoprotein (lectin-like) receptor 1	mCG130118.1	8.9	2.3	unknown
ubiquitin D	mCG23392.1	7.1	3.9	unknown

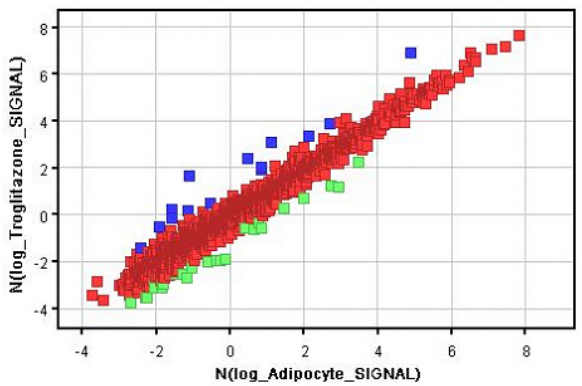

Fig 4. Scatter plot of signals detected by cRNAs prepared from preadipocytes and adipocytes.
Signals were detected using the AB 1700 chem-iluminescent microarray analyzer and normalized. Normalized (log adipocyte signal); normalized (log troglitazone-treated adipocyte signal); Blue, red and green colors indicate over 2.0 fold, between 2.0 fold and -2.0 fold, under -2.0 fold respectively.

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