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Proteomic and genomic analysis in rat brown adipose tissue for mining biomakers

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ABSTRACT

Chapter1. Gender Difference in Proteome of Brown Adipose Tissues between Male and Female Rats Exposed to a High Fat Diet

Although the importance of gender as a key determinant in health and illness has been recognized for a long time, systematic studies of gender differences in medicine are still lacking. We hypothesized that interscapular brown adipocyte tissue (BAT), is not only a key tissue contributing to energy expenditure, but also regulates diet-induced thermogenesis, and may be an ideal target for studying gender differences in obesity development in response to a high fat diet (HFD). We therefore performed differential proteome analysis of BAT from lean and obese rats of both genders fed a HFD using 2-DE combined with MALDI-TOF-MS. When exposed to a HFD, male rats gained more body weight with increased values of plasma biochemical parameters than did female rats. Among 595 matched spots, 48 differentially expressed identified spots showed significant gender differences, whereas 7 proteins showed no gender differences, but did show a HFD response. Proteomic investigations into gender-dimorphic protein modulation in BAT may provide conclusive results showing higher expression of numerous proteins involved in thermogenesis and fat oxidation as well as lower expression of proteins contributing to fat synthesis in female rats than in male rats.

Chapter2. Gender-dependent gene expressions in brown adipose tissue of lean and obese rats fed a high fat diet

In the present study, we identified the interscapular brown adipose tissue (BAT) genes showing differential expression using DNA microarray analysis in order to better understand gender-different gene regulation as well as molecular abnormalities in diet-induced obesity. To understand the detailed changes in the gene expression profiles in BAT caused by HFD feeding, we extracted and summarized the genes that were up- or down-regulated by more than 1.5-fold between the genders. In this analysis, significant global changes were observed at the mRNA levels between the genders as well as lean and obese rat BAT rendered by a HFD. We report for the first time that a series of genes, which might be involved in fatty acid oxidation and thermogenic regulation, were more highly expressed in females than males. These results allowed us to conclude that compared to males, females have greater fat clearing capacity through the activation of genes encoding enzymes of fat oxidation. In addition, we also found that females have higher thermogenic capacity due to increased expressions of genes involved in energy expenditure. In conclusion, the microarray data of gender dimorphism in BAT will prove valuable in improving gender awareness in the health care system and for the development of evidence-based gender specific clinical recommendations

Ⅰ. Introduction

1. Gender Difference in Proteome of Brown Adipose Tissues between Male and Female Rats Exposed to a High Fat Diet

A broader gender-sensitive knowledge would provide the basis for specific evidence-based interventions allowing prevention, treatment of many diseases by matching the different needs of women and men such as development of gender medicine as an essential health policy (Kautzy-Willer and Handisurya, 2009). Although many factors such as social and cultural environments are considered as important causes of obesity between male and female, biological differences are more profound (Sweeting 2008).

To date, numerous studies have been conducted in an effort to address sex difference in relation to obesity, including difference in body fat distribution (Blouin et al. 2008; Nguyen et al. 1996; Blaak, 2001; Lladó et al., 2000; 2002; Shi et al. 2009; Zillikens et al. 2008, Schousboe et al.2003), physical activity (Kautzy-Willer and Handisurya, 2009), metabolic rate (Arciero et al. 1993; Tarnopolsky et al. 1993; Komi and Karlsson, 1978; Henderson et al. 2008), oxidative capacity (Rodríguez and Palou, 2004), sex hormones (Roca et al. 1999; Bjoentörp 1997; Rodríguez et al, 2002; Allan et al. 2008), energy expenditure (Quevedo et al., 1998; Rodríguez et al., 2001; Valle et al. 2005; Macotela et al., 2009; Wang et al., 2009), adipokine signaling (Matsuda et al. 2005; Fujiki et al., 2006; Havel et al., 1996, Nature Medicine), and appetite regulation (Clegg et al. 2003; Wang et al. 2009, Frank et al. 2010; Magkos et al. 2009). However, the role played by gender in susceptibility to obesity is not fully understood. A line of evidence has demonstrated that males have a higher susceptibility to becoming obese, compared with females (Hong et al. 2009; Legato, 1997; Gayle et al. 2006; add more references). However, some

contradictory results have been found in the literature that may be explained by differences between rat strains and gender, as well as differences in the nutritional state of the animals (Kautzy-Willer and Handisurya, 2009; Rodríguez and Palou, 2004; Català-Niell et al. 2008; Rodríguez-Cuenca et al. 2002; Justo et al. 2005; Valle et al. 2007).

Several lines of evidence suggest that females have greater energy expenditure than males due to the higher thermogenic activation (Valle et al. 2005), greater oxidative capacity with more effective antioxidant machinery (Català-Niell et al. 2008), and greater respiratory and phosphorylative capacities in mitochondria (Rodríguez-Cuenca et al. 2002; Justo et al. 2005; Valle et al. 2007). In this regard, BAT, as a key tissue contributes to energy expenditure, may be an ideal target for allowing gender difference in obesity development in response to HFD.

To date, several proteomics studies have been conducted in an effort to reveal gender differences in protein expression patterns in blood (Miike et al. 2010; Eidelman et al. 2010), muscle (Metskas et al., 2010), kidney (Amelina and Cristobal, 2009), brain (Martins-de-Souza et al. 2010), and sperm (Shetty et al. 1999). However, to the best of our knowledge, no comparative proteomic study in biofluids or metabolic organ tissues of lean and obese animal models for examination of protein abundance and/or regulation patterns between genders has been conducted. Despite its important function in diet-induced thermogenesis, studies about BAT proteome with respect to obesity have not been conducted with the exception of two recent published results (Schmid et al., 2004; Joo et al. 2011).

In the present study, we therefore performed differential proteome analysis of interscapular
BAT to determine sex-specific susceptibility to obesity between male and female rats, and to find proteins determining phenotypic differences between the

genders.

2. Gender-dependent gene expressions in brown adipose tissue of lean and obese rats fed a high fat diet

There is an increasing evidence that clinical manifestation of various metabolic diseases such as diabetes and obesity are influenced by genders (Kautzky-Willer and Handisurya, 2009; Legato, 1997; Power and Schulkin, 2008). Thus, knowledge of gender dimorphism is important because nutritional recommendations on the basis of data collected by using predominantly male subjects may not be valid for women. Although the importance of sex as a key determinant in health and illness has been recognized for a long time, systematic studies of sex differences in medicine are still lacking (Zillikens et al., 2008).

It has been demonstrated that sexual dimorphism in obesity stems from many factors, including dissimilarity in the patterns of physical activity (Kautzky-Willer and Handisurya, 2009), metabolic rate (Arciero et al., 1993; Henderson et al., 2008; Komi and Karlsson, 1978; Tarnopolsky et al., 1995), energy expenditure (Quevedo et al., 1998; Rodriguez et al., 2001; Valle et al., 2005), oxidative capacity (Rodriguez and Palou, 2004b), sex hormones (Allan et al., 2008; Bjorntorp, 1997), as well as fat deposition and fat mobilization (Power and Schulkin, 2008).

Several earlier studies suggest that energy expenditure in females is greater than that of male due to higher thermogenic activation (Valle et al., 2005), greater oxidative capacity with more effective antioxidant machinery (Catala-Niell et al., 2008), as well as greater respiratory and phosphorylative capacities in mitochondria (Justo et al., 2005; Rodriguez-Cuenca et al., 2002; Valle et al., 2007). Our recent studies also verified these results by proteomic approach (Choi et al. 2011, Liu et al. 2011, Wang et al. 2011). In this regard, BAT, as a key tissue contributing to energy expenditure, may be an ideal target for allowing gender difference in obesity development in response to HFD.

DNA microarray has been used to screen the genome-wide expression by enabling analysis of thousands of gene at one time in a single experiment, thereby indentifying a overall assessment of obesity-related gene expression levels (Joo and Yun, 2011; Klaus and Keijer, 2004; Ross et al., 2002).

Indeed, most cases of obesity are polygenic and represent interaction between multiple genes and the environment, wherein diet is a major component. For this reason, gene expression profiling may be helpful in the search for key player genes determining phenotypic differences between the genders. We hypothesized that differences in gene expression in adipocytes is likely to contribute to this response.

To date, several genomic studies have been conducted in an effort to identify genes for which expression levels are specifically regulated in BAT, in response to many stimuli such as exposure to cold or high fat feeding (Boeuf et al., 2001; Joo and Yun, 2011; Unami et al., 2004; Yan et al., 2006). However, to the best of our knowledge, no comparative study to understand the detailed changes in the gene expression profiles in BAT caused by HFD feeding has been conducted.

In the present study, to seek the answers to numerous questions about gender-different gene regulation as well as molecular abnormalities of diet-induced obesity, we attempted to identify the BAT genes differentially expressed using DNA microarray analysis of BAT of both genders.

II. Materials and methods

1. Animals and breeding conditions

Male and female SLC Sprague-Dawley (SD) rats were purchased from Daehan Experiment Animals (Seoul, Korea) at 5 weeks of age and were housed one per cage in a temperature (23±2° C) and humidity (55%)-controlled room with a 12 hr light/dark cycle. Rats were provided free access to standard chow

and tap water for an adaption period of 1 week. Male and female rats were randomly divided into two groups, with 20 rats fed an ND (12% calories from fat; control group) and 40 rats fed an HFD (45% calories from fat), respectively. Rat feeds were purchased from Feed Korea Lab (Hanam, Korea); the dietary composition of these feeds is shown in Supplementary Table 2. All rats and feeds were weighed every week for 8 weeks. Rats were food deprived for at least 12 hr before sacrifice and were anesthetized by 3% diethyl ether. These experiments were approved by the Committee for Laboratory Animal Care and Use of Daegu University. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

1.1. Blood plasma samples

Blood samples were obtained by resection at the end of the rat tail under anesthesia and collected into EDTA-tubes (BD, Franklin Lakes, NJ, USA). Plasma was separated by centrifugation (3,000×*g*, 10 min), followed by storage at −80℃ until further analysis. Protein concentration of plasma was determined by the Bradford method (Bradford, 1976) using protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA).

1.2. Plasma biochemical parameters

Using plasma of ND-fed rats (n=7) and HFD-fed rats (n=7) in male and female rats, respectively, plasma biochemical parameters were measured. Commercial kits purchased from Bio Clinical System Corporation (Anyang, Korea) were used for enzymatic measurement of total cholesterol and high density lipoprotein (HDL) cholesterol in plasma, and enzymatic kits of Asan Phamaceutical (Seoul, Korea) were used for determination of triglyceride (TG) and glucose. An enzymatic fatty acid quantification kit (Abcam, Cambridge, UK) was used for quantification of free fatty acids (FFA). A Rat Insulin kit (Millipore Co., Billerica,

MA, USA) was used for measurement of plasma insulin levels, and leptin levels were gauged by use of the Rat Leptin kit (Millipore), which is a sandwich enzyme-linked immunosorbent assay (ELISA) system. Sex hormone levels were measured using a Rat Estrogen ELISA kit (Cusabio Biotech. Co., LTD, Wuhan, Hubei, China) and Rat Testosterone ELISA kit (Cusabio Biotech). Measurements were performed according to the manufacturer's instructions.

1.3. Preparation of BAT samples for 2-DE

Interscapular BAT of rats in each group was excised immediately after the rats were anesthetized with diethyl ether after an overnight fasting. And then the resulting tissues were washed with a cold saline solution. BAT was pulverized under liquid nitrogen and stored at −80 ° C. Tissues were lysed in 200 μl rehydration buffer solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 1 mM PMSF, 2% IPG buffer (Ampholyte 3/10, Bio-Rad), and a trace of bromophenol blue. A homogenizer (PT 1200E, Kinematica Ltd., Luzern, Switzerland) was used on ice. Extracts were centrifuged at 13000×g for 20 min; the supernatant was then stored at −80° C until analysis. Protein content of adipose tissues was determined by the Bradford method (Bradford, 1976) using Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA).

1.4. 2-DE analysis

2-DE was performed three times using pooled BAT protein samples from seven rats per group, which consisted of the control group, who were fed an ND, and the obese group, who were fed an HFD in both male and female rats. Here, 2-DE experiments were conducted using previous methods (Choi et al. 2010). Briefly, immobilized pH gradient (IPG)-isoelectric focusing (IEF) of BAT samples was performed on pH 3-10 and 18 cm IPG DryStrips (GE healthcare, Buckinghamshire, UK) in a PROTEIN IEF cell (Bio-Rad) using the protocol recommended by the manufacturer. IPG strips were rehydrated passively for 24

h in strip holders with 350 μL of rehydration solution, which included 7 M urea (Bio Basic, Ontario, Canada), 2 M thiourea (Sigma, St. Louis, MO, USA), 4% CHAPS (Bio Basic), 1 mM PMSF (Sigma), 20 mM DTT (GE Healthcare), and 2% IPG buffer (Bio-Rad), and BAT protein of 25 μg without albumin depletion. IEF was executed as follows: 15 min at 250 V, 3 h at 250-10,000 V, 6 h at 10,000 V, and then held at 500 V until ready to run the second dimension. After focusing, the gel strips were equilibrated in a solution containing 6 M urea, 2% SDS (Generay Biotech, Shanghai, China), 1% DTT, 30% glycerol (Bio Basic), and 50 mM Tris-HCl (pH 6.8) for 20 min, followed by further incubation in the same solution, except for replacement of DTT with 2.5% iodoacetamide (Bio-Rad) for an additional 20 min. Gel strips were then placed onto a 20×20 cm 12% polyacrylamide gel for resolution in the second dimension. Fractionation was performed with the Laemmli SDS-discontinuous system, at a constant voltage of 15 mA per gel for 14 h. For image analysis and peptide mass fingerprinting (PMF), a total of 12 gels, including 3 gels per each group with separated protein were visualized by silver staining. Silver staining was performed as follows. Gels were fixed over a period of 30 min in 50% ethanol (DUKSAN pure Chemicals, Ansan, Korea) and 5% acetic acid (DUKSAN pure Chemicals), followed by 10 min in 30% ethanol, and water-washed for 5 min three times. Gels were sensitized for 10 min in 0.02% sodium thiosulfate (Sigma), followed by 0.5 min water washes three times, and incubated for 25 min in 0.3% silver nitrate (Kojima Chemicals, Sayama, Japan). After two 0.5 min water washes, proteins were visualized with developing solution (3% sodium carbonate (DUKSAN, pure Chemicals), 0.02% sodium thiosulfate, 0.05% formalin (DC Chemicals, Incheon, Korea), and stopped with 6% acetic acid.

1.5. Image acquisition and data analysis

Gels were imaged on a UMAX PowerLook 1120 (Maxium Technologies, Akron, OH, USA) and comparison of images was performed using modified ImageMaster

2-D software V4.95 (GE Healthcare). A reference gel was selected from gels of the normal group and detected spots from the other gels were matched with those in the reference gel. Relative optical density and relative volume were also calculated for the purpose of correction for differences in-gel staining. Each spot intensity volume was processed by background subtraction and total spot volume normalization; the resulting spot volume percentage was used for comparison.

1.6. Peptide mass fingerprinting (PMF)

For protein identification, protein spots were excised, digested with trypsin (Promega, Madison, WI), mixed with α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid, and subjected to matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) analysis (Ettan MALDI-TOF Pro, GE healthcare). Spectra were collected from 350 shots per spectrum over an *m/z* range of 600-3000 and calibrated by two point internal calibration using trypsin auto-digestion peaks (*m/z* 842.5099, 2211.1046). Peak list was generated using the Ettan MALDI-TOF Pro Evaluation Module (ver 2.0.16). The threshold used for peak-picking was as follows: 5,000 for minimum resolution of monoisotopic mass, 2.5 for S/N. The search program MASCOT (Mascot Sever 2.3), developed by The Matrixscience (http://www.matrixscience.com), was used for protein identification by PMF. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a fixed modification, oxidation (Met) as a variable modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. MASCOT probability-based MOWSE (molecular weight search) score was calculated for PMF. Protein score is -10*Log (P), where P is the probability that the observed match is a random event, and greater than 61 are significant ($p<0.05$).

1.7. Immunoblot analysis

Levels of six proteins identified on a 2-DE protein map together with 3 BAT proteins were confirmed by immunoblot analysis. Tissue lysates were prepared with RIPA buffer (Sigma-Aldrich), homogenized, and centrifuged at 12000×*g* for 20 min. The extract was diluted in 5X sample buffer (50 mM Tris of pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue) and heated for 5 min at 95℃ before SDS-polyacrylamide gel electrophoresis (PAGE) gel of 6, 8, 10, or 12%. After electrophoresis, they were transferred to a polyvinylidene difluoride (PVDF, Santa Cruz Biotechnology, Santa Cruz, CA, USA) membrane and blocked for 1 h with TBS (tris-buffered saline)-T buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 containing 5% skim milk). The membrane was rinsed in three changes of TBS-T buffer, followed by incubation for 1 h with a 1:1000 dilution of primary polyclonal antibody (anti-CK; Frontier, anti-FAS; Cell Singnaling, anti-HADHA; Invitrogen and anti-β-actin; Santa Cruz Biotechnology) in TBS-T buffer containing 1% skim milk. After three washes, the membrane was incubated for 1 h with horseradish peroxidase-conjugated anti-goat IgG, anti-mouse IgG, or anti-rabbit IgG secondary antibody (1:1000, Frontier) in TBS-T buffer containing 1% skim milk and developed using enhanced chemiluminescence (ECL; GE healthcare). Immunoblot analysis was performed by scanning with a UMAX PowerLook 1120 and digitalized using image analysis software (KODAK 1D, Eastman Kodak, Rochester, NY, USA).

1.8. Determination of the relative mitochondrial copy number

Genomic DNA was isolated and purified from approximately 20 mg of frozen interscapular BAT employing the G-spin Genomic DNA Extraction Kit (Intron). The mitochondrial DNA (mtDNA) content relative to the peroxisome proliferator-activated receptor-γ coactivator 1 (PGC1-α) gene was measured performing real-time RT-PCR (Stratagene Mx3000p QPCR system,

San Diego, CA, USA) (Ciapaite et al., 2007). Primers for mtDNA were designed using forward 5′-ACACCAAAAGGACGAACCTG-3′ and reverse 5′-ATGGGGAAGAAGCCCTAGAA-3′ and for PGC1-α forward 5′-ATGAATGCAGCGGTCTTAGC-3′ and reverse 5′-AACAATGGCAGGGTTTGTTC-3′ as described previously (Kim et al. 2011).

1.9. Quantitative real-time RT-PCR analysis

Total RNA was extracted from interscapular BAT using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA); 1 μg of isolated RNA was reverse-transcripted using the cDNA Reverse Transcription Kit. Transcript levels of genes were determined using quantitative real-time PCR using SYBR Green I Dye Chemistry, according to the manufacturer's protocol. Primer for UCP1 designed using forward 5′-GGGACCTACAATGCTTACAG-3′ and reverse 5′-GGTCATATGTCACCAGCTCT-3′ and for GAPDH forward 5′-GGTCTCGCTCCTGGAAAGA-3′ and reverse 5′-GTATGACTCCACTCACGGCAA-3′. Reaction mixtures were preheated at 95°C for 10 min; temperature conditions were as follows for 45 cycles; melting at 95°C for 30 sec, annealing at 60°C for 1 min, and elongation at 72°C for 1 min. Sequences of used primer sets are listed in Table 4. Transcript levels of each gene were normalized to those of GAPDH.

2. Animals and breeding conditions

Five-week-older male and female SLC Sprague-Dawley (SD) rats were purchased from Daehan Experimental Animals (Seoul, Korea) and were housed one per cage in a temperature (23±2° C) and humidity (55%)-controlled room with a 12 hr light/dark cycle throughout the experimental period. Rats were allowed free access to standard chow and tap water for an adaption period of one week for acclimatization before experiments. After one week, male and

female rats were randomly separated into two groups, with 20 rats fed an ND (12% calories from fat; control group) and 40 rats fed an HFD (45% calories from fat), respectively. Rat feeds were bought from Feed Korea Lab (Hanam, Korea). All rats and feeds were weighed every week during 8 weeks. At least, rats were food deprived for 12 hr before sacrifice and were anesthetized by 3% diethyl ether. These experiments were approved by the Committee for Laboratory Animal Care and Use of Daegu University. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

2.1. RNA preparation

According to the manufacturer's instructions, total RNA was extracted using Trizol (Invitrogen Life Technologies, Carlsbad, USA), and purified using RNeasy columns (Qiagen, Valencia, USA). After processing with DNase digestion, clean-up procedures, RNA samples were quantified, aliquotted, and stored at -80° C until use. For quality control, purity of the total RNA was determined by the ratio of absorbance readings at 260 and 280 nm, the ratio of which fell within the range of 1.8-2.0. Integrity of RNA was analyzed by denaturing agarose gel electrophoresis, with the 28S rRNA band appearing approximately twice as intense as the 18S rRNA band and analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA).

2.2 Labeling and purification

Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, USA) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo(dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop,

Wilmington, USA).

2.3. Hybridization and data export

750 ng of labeled cRNA samples were hybridized to each rat-12 expression bead array for 16-18 h at 58° C, according to the manufacturer's instructions (Illumina, Inc., San Diego, USA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions.

2.4. Raw data preparation and statistical analysis

The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using software provided by the manufacturer (Illumina GenomeStudio v2009.2 (Gene Expression Module v1.5.4). Array data were filtered by detection p-value<0.05 (similar to signal to noise) in at least 50% of samples. We applied a filtering criterion for data analysis; higher signal value was required in order to obtain a detection p-value≤0.05. Selected gene signal value was transformed by logarithm and normalized by a quantile method. Statistical significance of the expression data was determined using ANOVA and the TukeyHSD test, in which the null hypothesis was that no difference exists between mean of groups in the data. False discovery rate (FDR) was controlled by adjustment of p value using the Benjamini-Hochberg algorithm. Gene-ontology analysis for a significant probe list was performed using PANTHER (http://www.pantherdb.org/panther/ontologies.jsp), using text files containing the Gene ID list and accession number of Illumina probe ID. Gene Set Enrichment Analysis (GSEA) was performed in order to determine whether an a priori defined set of genes showed differential patterns in both biological

processes and molecular function states. One-tail Fisher Exact was adopted for measurement of gene-enrichment in annotation terms. Hierarchical and K-means clustering was performed using complete linkage with a Euclidian metric. GenomeStudio v2009.2 software was used for quantification and image analysis of mRNA data. R scripts were used for all other analytical processes.

2.5. Quantitative real-time RT-PCR analysis

Total RNA was isolated from BAT samples with Trizol (Qiagen Inc., Valencia, USA), and was then cleaned with ribonuclease-free deoxyribonuclease and the RNeasyMini kit (Qiagen). Samples were then processed according to the manufacturer's instructions. Quality of the RNA was analyzed on a 1% (w/v) agarose gel and was concentrated by a Qubit quantitation system (Invitrogen, Eugene, USA). Briefly, reverse transcription of 2 μg RNA was carried out using the iScript cDNA synthesis kit, according to the supplier's protocol. According to the manufacturer's protocol, quantitative real-time PCR was performed using the Applied Stratagene mx 3000p QPCR System (San Diego, USA). The oligonucleotide sequences used were presented in Table 1.

3. Statistical analysis

All experimental results were compared by one-way analysis of variance (ANOVA) using the Statistical Package of Social Science (SPSS, version 14.0K) program; data are expressed as the mean ± SEM. Group means were considered significantly different at $p<0.05$, as determined by the technique of protected least-significant difference (LSD) when ANOVA indicated an overall significant treatment effect ($p<0.05$).

III. Results and Discussion

Chapter 1. Gender difference in proteome of brown adipose tissue between male and female rats exposed to a high fat diet

1. Results

1.1. HFD-induced phenotypes in male and female rats

Male and female rats were randomly divided into two groups, with 20 rats fed an ND and 40 rats fed an HFD, respectively. At the beginning, body weight and food intake of ND and HFD rats were nearly identical in male and female rats. However, body weight in HFD-fed rat were significantly higher ($p<0.05$) than those in ND-fed counterparts at all subsequent time points in both male and female rats after 2 weeks (Fig. 1A). Total body weight gain per food intake of HFD-fed male rats was higher in females by an average of approximately 30 % ($p=0.03\text{x}10^{-6}$) (Fig. 1B). Next, various biochemical parameters were measured in each group. As a result, the concentration of plasma HDL-cholesterol was significantly lower in HFD rats, compared with ND rats (males, $p=0.004$; females, $p=0.018$), whereas total cholesterol (males, $p=0.032$; females, $p=0.044$), TG (males, $p=0.049$; females, $p=0.028$), and FFA (males, $p=0.049$) levels were remarkably higher in HFD rats, compared with those of ND rats in both males and females (Table 1). In particular, plasma glucose levels were found to be higher in males, compared with female rats (ND, $p=2\text{x}10^{-4}$). The levels of plasma insulin and leptin in seven rats were also determined in ND and HFD rats (Table 1). Results revealed that average insulin and leptin levels of HFD rats were significantly higher than in ND rats (insulin in males, $p=0.03$; leptin in males, $p=2\text{x}10^{-5}$; leptin in females, $p=0.013$), and they were remarkably higher in male rats than in female rats (insulin in ND rats, $p=4\text{x}10^{-4}$; leptin in ND rats, $p=0.011$; leptin in HFD rats, $p=0.007$). We also investigated the plasma concentration of sex hormones, estrogen and testosterone. The

concentration of estrogen was significantly higher in HFD rats, compared with ND rats, in both males and females (males, p=0.01; females, p=0.003). However, testosterone concentration was higher in ND rats, compared with HFD rats, only in male rats (Table 1). These collective results prompted us to perform further proteomic studies.

1.2. Proteomic analysis of BAT proteins

2-DE-based proteomic experiments were conducted three times using pooled BAT samples from seven rats per group for examination of differential expression of proteins between gender and diet. For effective proteomic analysis, whole proteins of low concentration (150 μg/gel) with abundant proteins were used. BAT proteins were separated by 2-DE using a pH 3-10 IEF strip for the first dimension and 12% (w/v) SDS-PAGE gel for the second dimension. Consequently, nearly 595 individual spots were detected, ranging from 6 to 240 kDa mass between pH 3 and 10 (Fig. 2). Based on high scores (p<0.05) and sequence coverage, a total of 55 proteins among approximately 595 spots were identified with high confidence by MALDI-TOF/MS and database searches (Table 2).

1.3. Gender-dependent expressions of BAT proteins in both ND and HFD rats

Image analysis and further statistical analysis allowed us to detect and identify 48 proteins whose expressions were significantly modulated in a gender-dependent manner, at least in ND or HFD rats. Those proteins were further classified into two groups based on their expression patterns in response to diet and gender. Group I categorized 23 proteins showing gender-difference with identical expression patterns in both ND and HFD rats. Of these, levels of 11 proteins were higher in males while those of 12 proteins were higher in females. Highly regulated proteins in males in both ND and HFD rats included electron-transfer flavoprotein (ETF), NADP-dependent malic enzyme (ME), asparagines

synthease (AS), NADH dehydrogenase (ubiquinine) flavoprotein 1 (NDFV1), zinc-binding alcohol dehydrogenase domain-containing protein 2 (ZADH2), glucose-related protein 75 (GRP75), cytosol aminopeptidase (CAP), methylcrotonoyl-CoA carboxylase beta chain (MCC-beta chain), T-complex protein 1 subunit gamma (TPSG), Aldose reductase (AR) and Perilipin-1 (PLIN) (Fig. 3 A).

Meanwhile, higher protein levels in females of both ND and HFD rats included glycerol kinase (GyK), glycerol-3-phosphate dehydrogenase (GPDH), phophoenolpyruvate carboxylase (PEPCK), succinyl-CoA ligase subunit beta 2, mitochondrial (Sucla2), fumarase, branched chain α-keto acid dehydrogenase complex (BCKADC), creatin kinase (CK), adipocyte plasma membrane-associated protein (APMAP), kinesin heavy chain isoform 5C (KHC), NADH dehydrogenase (ubiquinone) Fe-S protein 1(NDUFS1), zinc binding alcohol dehydrogenase domain-containing protein1 (ZADH1) and enolase 1(ENO1) (Fig. 3 B).

Among those proteins, KHC exhibited the greatest gender difference in both ND and HFD rats. Interestingly, 4 proteins showed gender-difference with opposite expression patterns in both ND and HFD rats; these included fatty acid synthase (FAS), carboxylesterase 3 (CES3), aldehyde dehydrase (AR), rCG55067 (Fig. 4). In addition, interestingly, as shown in Fig. 5, 15 proteins showed gender difference only in ND while 6 were gender-dependently expressed only in HFD rats (Fig. 5 A).

1.4. HFD-responsive but not gender-dependently expressed BAT proteins

As presented in Fig. 5 B, we also found that a total of 7 proteins showed no gender-difference, but exhibited significantly HFD-responsive regulation in both male and females. Of these, levels of 2 proteins were higher in HFD-fed rats, whereas those of 5 proteins marked lower in HFD rats compared to ND rats.

1.5. Validation of the results from proteomic analysis using immunoblot analysis

Although our proteomic data indicated differential protein expression between male and female rats, we could not exclude the possibility of technical errors and artificial effects in proteomic analysis. To verify this issue, levels of 3 proteins of interest (CK, FAS, HADHA) together with 6 metabolically important proteins in BAT (e.g. PPAR γ, GLUT4, UCP1, CPT1, AMPK, ACC) were investigated by immunoblot analysis. As shown in Fig. 6, protein levels of 3 proteins were in line with those from proteomic analysis showing gender- or HFD-responsive regulation. Levels of UCP1 and other 5 proteins of metabolic importance also showed significant gender-difference and showed HFD responsive regulation patterns (Fig. 7).

In order to compare thermogenic activity, mitochondrial content, PGC1-α as well as UCP1 protein and mRNA levels of UCP1 in BAT of ND and HFD-fed rats were determined. As shown in Fig. 8, all were higher in females than males in both diet conditions.

488 **Table 1.** Plasma biochemical parameters in each group[1)]
489

Parameter	Male	Female	Male	Female
	ND		HFD	
Glucose (mg/ml)	2.08±0.18**†	1.64±0.1†	2.39±0.24	2.21±0.58
TG (mg/ml)	0.49±0.09†	0.47±0.07†	0.71±0.23	0.67±0.19
Total cholesterol (mg/ml)	0.36±0.16†	0.32±0.2†	0.6±0.21	0.54±0.16
HDL cholesterol (mg/ml)	0.52±0.11††	0.51±0.09†	0.32±0.11	0.35±0.12
Free fatty acid (μmol/ml)	1.78±0.2†	1.79±0.14	2.05±0.26	1.97±0.25
Leptin (ng/ml)	6.62±2.62*††	2.77±2.15†	21±4.28**	11.2±6.51
Insulin (ng/ml)	1.67±0.48**†	0.57±0.3	2.43±0.65	1.5±1.27
Estrogen (ng/ml)	5.56±0.55**††	8.3±0.78††	6.39±0.47**	10.83±1.44
Testosterone (ng/ml)	1.95±1.85	0.31±0.07	0.92±0.56**	0.35±0.1

[1)] Levels of biochemical parameters were averaged in each group and expressed as a mean ±SEM of seven separate experiments. [1)] Statistical significance between male and female rats was determined by a t-test, where p value is $^{*}p<0.05$ and $^{**}p<0.01$ (male ND vs. female ND in the [ND, Male] column; male HFD vs. female HFD in the [HFD, Male] column). [1)] Significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$ (male ND vs. male HFD in the [ND, Male] column; female ND vs. female HFD in the [ND, Female] column).

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491

492 **Table 2.** Composition High Fat Diet and Normal Diet used in this study.

493

Composition by weight, g/kg		
Ingredient	LFD	HFD
Casein	200	200
Cornstarch	150	155
Sucrose	500	50
Dextrose	0	132
Cellulose	50	50
Soybean oil	0	25
Corn oil	50	0
Lard	0	175
Mineral mix[1)]	35	35
Vitamin mix[2)]	10	10
TBHQ[3)]	0	0.014
DL−Methionin	3	0
L−cystine	0	3
Choline bitartrate	2	2.5
Composition by calories, %		
Protein	21	20
Carbohydrate	68	35
Fat	12	45
Total	**3902 kcal/kg**	**4776 kcal/kg**

[1)]Mineral mix; sucrose (118.03 g/kg), calcium phosphate dibasic (500 g/kg), sodium chloride (74 g/kg), potassium citrate (220 g/kg), potassium sulfate (52 g/kg), magnesium oxide (24 g/kg), magnesium oxide (24 g/kg), manganous carbonate (3.5 g/kg), ferric citrate (6 g/kg), zinc carbonate (1.6 g/kg), cupric carbonate (0.3 g/kg), potassium iodate (0.01 g/kg), sodium selenite (0.01 g/kg), chromium potassium sulfate (0.55 g/kg).

[2)]Vitamine mix, sucrose (981.15 g/kg), thiamine (0.6 g/kg), vitamin E acetate (500 IU/g; 10 g/kg), niacin (3 g/kg), calcium pantothenate (1.6 g/kg), vitamin B12 (0.1%; 1 g/kg), vitamin A palmitate (500,000 IU/g; 0.8 g/kg), pyridoxine (0.7 g/kg), riboflavin (0.6 g/kg), vitamin D3 (400,000 IU/g; 0.25 g/kg), folic acid (0.2 g/kg), menadione sodium bisulfate (0.08 g/kg), biotin (0.02 g/kg).

[3)]TBHQ, tert−butylhydroquinone.X

Table 3. Differentially regulated and major BAT proteins in male and female rats identified through proteomic analysis.

No. in gel	Protein	Acc. No. [a]	Theoretical MW (kDa)	Theoretical PI	Alterations (Vol %) [b]				Cover-age (%) [c]	Score [d]
					Male	Female	Male	Female		
					Normal		HFD			
1	electron transfer flavoprotein subunit alpha precursor (ETF precursor)	gi|57527204	35.2	8.62	1.08±0.13	0.81±0.16[a]	1.03±0.04[b]	0.78±0.1	49	106
2	Dihydrolipoyl dehydrogenase precurso (DLD precursor)	gi|40786469	54.5	7.96	0.73±0.06[c]	0.66±0.09	0.83±0.05[b]	0.71±0.05	43	151
3	M2 pyruvate kinase-like isoform 1 (PKM2-like isoform 1)	gi|293350511	58.3	7.15	0.56±0.05	0.32±0.03[aa]	0.57±0.06	0.47±0.08[dd]	44	163
4	phosphoenolpyruvate carboxykinase (PEPCK)	gi|38454296	70.1	6.09	0.15±0.01	0.27±0.02[aa]	0.18±0.04[b]	0.30±0.04	52	321
5	Sucla2 protein	gi|187469277	47.7	6.06	0.19±0.02	0.29±0.02[a]	0.24±0.02[b]	0.36±0.05	50	218
6	Acly protein	gi|71680955	120.5	7.13	0.12±0.03[c]	0.06±0.01[a]	0.21±0.04	0.15±0.04[d]	36	331
7	T-complex protein 1 subunit beta (TPSB)	gi|54400730	57.7	6.01	0.12±0.04	0.10±0.04	0.16±0.01[b]	0.10±0.02	54	221
8	Tu translation elongation factor (EFTu)	gi|157820845	44	7.65	0.10±0.01[c]	0.08±0.01[a]	0.06±0.02	0.06±0.01	41	116
9	T-complex protein 1 subunit gamma (TPSG)	gi|40018616	61.1	6.23	0.10±0.00	0.06±0.01[aa]	0.10±0.01[b]	0.07±0.00	43	164

(Continued)

10	Branched chain keto acid dehydrogenase E1 (BCKDH)	gi|165971320	43.4	6.41	0.09±0.03	0.17±0.02[a]	0.10±0.02[b]	0.18±0.03	28	62
11	pyruvate dehydrogenase E1 component subunit alpha (PDA)	gi|109503594	44.8	8.49	0.08±0.00	0.06±0.01[a]	0.11±0.02	0.09±0.03	47	133
12	pyruvate carboxylase (PC)	gi|929988	130.3	6.25	0.15±0.05	0.27±0.04[a]	0.14±0.04	0.14±0.04[d]	40	344
13	enolase 3 (ENO3)	gi|109468300	54.3	5.81	0.04±0.02	0.05±0.02	0.06±0.01[b]	0.09±0.01[d]	42	101
14	Epoxide hydrolase 2 (sEH2)	gi|55716049	62.9	5.76	0.06±0.02	0.08±0.01	0.07±0.01[b]	0.04±0.00	35	195
16	kinesin heavy chain isoform 5C (KHC)	gi|157819777	109.6	5.86	0.01±0.1	0.08±0.01[aa]	0.01±0.00[bb]	0.06±0.01	14	61
17	grp75	gi|1000439	73.9	5.87	0.25±0.01	0.19±0.02[a]	0.26±0.03[b]	0.13±0.00[d]	40	191
18	adipocyte plasma membrane-associated protein (APMAP)	gi|77735352	42.2	5.64	0.09±0.02	0.14±0.01[a]	0.08±0.01[b]	0.14±0.01	57	242
19	methylcrotonoyl-CoA carboxylase beta chain (MCC-beta chain)	gi|58865926	61.9	8.56	0.12±0.01	0.08±0.01[a]	0.13±0.02[b]	0.08±0.01	55	208
20	N-acetylgalactosamine kinase (NAGK)	gi|62078569	51.1	6.04	0.02±0.00[c]	0.02±0.00	0.05±0.00[b]	0.02±0.00	30	90
21	creatine kinase B-type (CK B-type)	gi|31542401	42.9	5.33	0.07±0.04	0.17±0.04[a]	0.11±0.04[b]	0.21±0.01	55	176
22	NADP-dependent malic enzyme (ME)	gi|266504	64.5	6.49	0.67±0.06[cc]	0.53±0.05[a]	0.41±0.03[b]	0.32±0.04[dd]	51	243

(Continued)

23	glycerol kinase (GyK)	gi|158186653	58.2	5.49	0.04±0.01^{c}	0.07±0.01^{a}	0.06±0.01^{b}	0.09±0.01	53	225
24	UDP-glucose 4-epimerase (GALE)	gi|162287387	38.6	6.02	0.09±0.02^{c}	0.10±0.03	0.03±0.01	0.03±0.01^{dd}	29	68
26	malate dehydrogenase (MHD)	gi|15100179	36.6	6.16	0.22±0.02^{c}	0.23±0.02	0.11±0.04	0.10±0.00^{d}	35	93
27	isocitrate dehydrogenase [NAD] subunit beta precursor (ICDH subunit beta precursor)	gi|55926203	42.6	8.89	0.23±0.03^{c}	0.24±0.03^{a}	0.11±0.04	0.10±0.00^{d}	48	167
28	mitochondrial long-chain enoyl-CoA hydratase/3-hydroxycyl-CoA dehydrogenase alpha-subunit (HADHA)	gi|510108	83.2	9.11	0.36±0.12^{c}	0.18±0.03	0.06±0.04	0.06±0.01^{dd}	39	180
30	short-chain specific acyl-CoA dehydrogenase (SCAD)	gi|11968090	45.2	8.47	0.45±0.06^{c}	0.39±0.06	0.58±0.02	0.59±0.09^{d}	40	145
31	phosphoglycerate mutase 1 (PGM1)	gi|114326546	28.9	6.67	0.23±0.04^{c}	0.20±0.02	0.13±0.03	0.13±0.01^{d}	68	172
32	serotransferrin precursor (TF precursor)	gi|61556986	78.5	7.14	0.07±0.02^{cc}	0.06±0.01	0.21±0.01	0.21±0.03^{dd}	48	297
33	voltage-dependent anion-selective channel protein 2 (VDAC2)	gi|13786202	32.3	7.44	0.14±0.04^{c}	0.14±0.04	0.03±.01	0.05±0.01^{d}	37	79
34	aldose reductase (AR)	gi|6978491	36.2	6.26	0.10±0.01^{c}	0.06±0.00^{a}	0.15±0.00^{bb}	0.09±0.01^{d}	40	115
35	EH domain-containing protein 1 (EHD1)	gi|169642494	62.7	6.46	0.22±0.00^{c}	0.18±0.01^{a}	0.19±0.01	0.16±0.05	37	144

(Continued)

36	acyl-CoA synthetase family member 2 precursor (ACSF2 precusor)	gi|77993368	68.6	8.39	0.19±0.03^{c}	0.12±0.01^{a}	0.11±0.01	0.11±0.03	44	153
37	glycerol-3-phosphate dehydrogenase 2 (GPDH)	gi|149047794	66.9	5.80	0.08±0.01^{cc}	0.11±0.01^{a}	0.05±0.01^{b}	0.08±0.00^{d}	58	243
38	Acyl-Coenzyme A dehydrogenase (ACD)	gi|56541110	71.1	8.94	0.20±0.03	0.11±0.02^{a}	0.27±0.04	0.22±0.00^{dd}	37	176
39	asparagine synthetase (AS)	gi|148747576	64.7	6.01	0.14±0.01^{cc}	0.11±0.00^{aa}	0.10±0.00^{b}	0.07±0.01^{d}	28	93
40	dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex (ACEF)	gi|78365255	67.6	8.76	0.15±0.01	0.22±0.02^{a}	0.17±0.03	0.17±0.03	26	85
41	cytosol aminopeptidase (CAP)	gi|58865398	56.5	6.77	0.11±0.00^{cc}	0.10±0.00^{aa}	0.14±.01^{bb}	0.10±0.00	46	171
42	Fumarase	gi|227665	54.7	9.14	0.07±0.01^{cc}	0.09±0.01^{a}	0.13±0.01^{bb}	0.16±0.00^{dd}	24	90
43	zinc-binding alcohol dehydrogenase domain-containing protein 2 (ZADH2)	gi|157817316	40.7	7.63	0.18±0.04	0.10±0.01^{a}	0.12±0.03^{bb}	0.06±0.01^{d}	22	74
45	zinc binding alcohol dehydrogenase, domain containing 1 (ZADH1)	gi|149025122	22.6	7.67	0.05±0.01	0.11±0.01^{aa}	0.04±0.01^{b}	0.06±0.00^{d}	39	78
46	G elongation factor (EF-G)	gi|293345129	86.6	6.07	0.02±0.00	0.04±0.00^{aa}	0.03±0.01	0.02±0.00^{dd}	29	203
48	unnamed protein product (PY02383)	gi|1334284	58	5.35	0.07±0.01^{cc}	0.14±0.03^{a}	0.15±0.01	0.15±0.07	29	88

(Continued)

49	isocitrate dehydrogenase 3 (NAD) gamma (ICDH3 gamma)	gi|149029908	41.2	9.08	0.09±0.01	0.05±0.01[a]	0.14±0.04	0.21±0.03[d]	35	87
50	NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1)	gi|149061921	36.8	5.89	0.09±0.01[c]	0.06±0.01[a]	0.06±0.01[b]	0.02±0.00[d]	62	178
51	Carboxylesterase 3 (CES3)	gi|57013350	62.3	6.10	0.07±0.03[cc]	0.16±0.02[a]	0.023±0.02[b]	0.17±0.02	27	89
52	Fatty acid synthase (FAS)	gi|2506136	27.5	5.96	0.03±0.01[c]	0.05±0.00[aa]	0.05±0.01[b]	0.03±0.01[d]	12	93
53	aldehyde dehydrogenase (AD)	gi|16073616	48.6	6.06	0.08±0.00[c]	0.07±0.00[a]	0.03±0.01[b]	0.07±0.001	30	80
54	perilipin-1 (PLIN)	gi|6981372	55.9	6.37	0.13±0.01	0.11±0.00[a]	0.13±0.01[b]	0.10±0.00	38	89
55	rCG55067	gi|149045627	98.7	7.01	0.11±0.01	0.09±0.01[a]	0.11±0.00[bb]	0.13±0.00[dd]	9	76
57	heat shock 70 kDa protein 4 (HSP70RY)	gi|24025637	94.7	5.13	0.03±0.01[c]	0.06±0.01[a]	0.05±0.00	0.05±0.00	33	178
58	NADH dehydrogenase (ubiquinone) Fe-S protein 1 (NDUFS1)	gi|149046009	74.3	5.74	0.08±0.02	0.14±0.01[a]	0.11±0.00[bb]	0.12±0.00	26	132
59	cytosolic non-specific dipeptidase (CNDP2)	gi|58219062	53.1	5.43	0.04±0.02[c]	0.08±0.01[a]	0.08±0.00	0.07±0.02	53	236
60	alpha-enolase isoform 1 (ENO1)	gi|158186649	47.4	6.16	0.02±0.00	0.07±0.00[aa]	0.01±0.00[b]	0.03±0.01[dd]	25	62
61	adenylate kinase 2 (AK2)	gi|77020256	25.7	7.01	0.06±0.03[c]	0.05±0.03	0.17±0.04[b]	0.09±0.01	34	64

[a]Acc. No. :NCBInr database accession number
[b]Statistical significance was determined by a t-test, where p-valus is *p<0.05 and **p<0.01:Nor Male vs. Nor Female [a]p<0.05 and [aa]p<0.01: HF Male vs. HF Female [b]p<0.05 and [bb]p<0.01 : Nor Male vs. HF Male [c]p<0.05 and [cc]p<0.01 : Nor Female vs. HF Female [d]p<0.05 and [dd]p<0.01. For each protein, the relative intensity was averaged and expressed as a mean± S.E.M of three separate experiments.
[C]Protein sequence coverage for the most probable candidate of known protein.
[d]Protein scores greater than 61 are significant ($p < 0.05$).

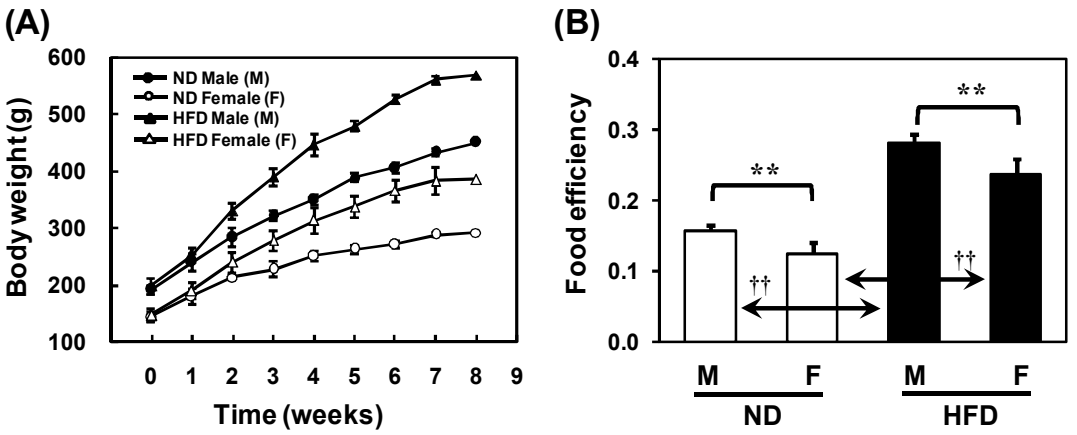

Fig. 1. Body weight (A) and food efficiency (B) in male and female rats fed ND and HFD. Data are presented as mean ±SEM in seven rats per group and were estimated using the ANOVA test. Statistical significance between male and female rats was determined by a t-test, where p value is $^{*}p<0.05$ and $^{**}p<0.01$ and significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$.

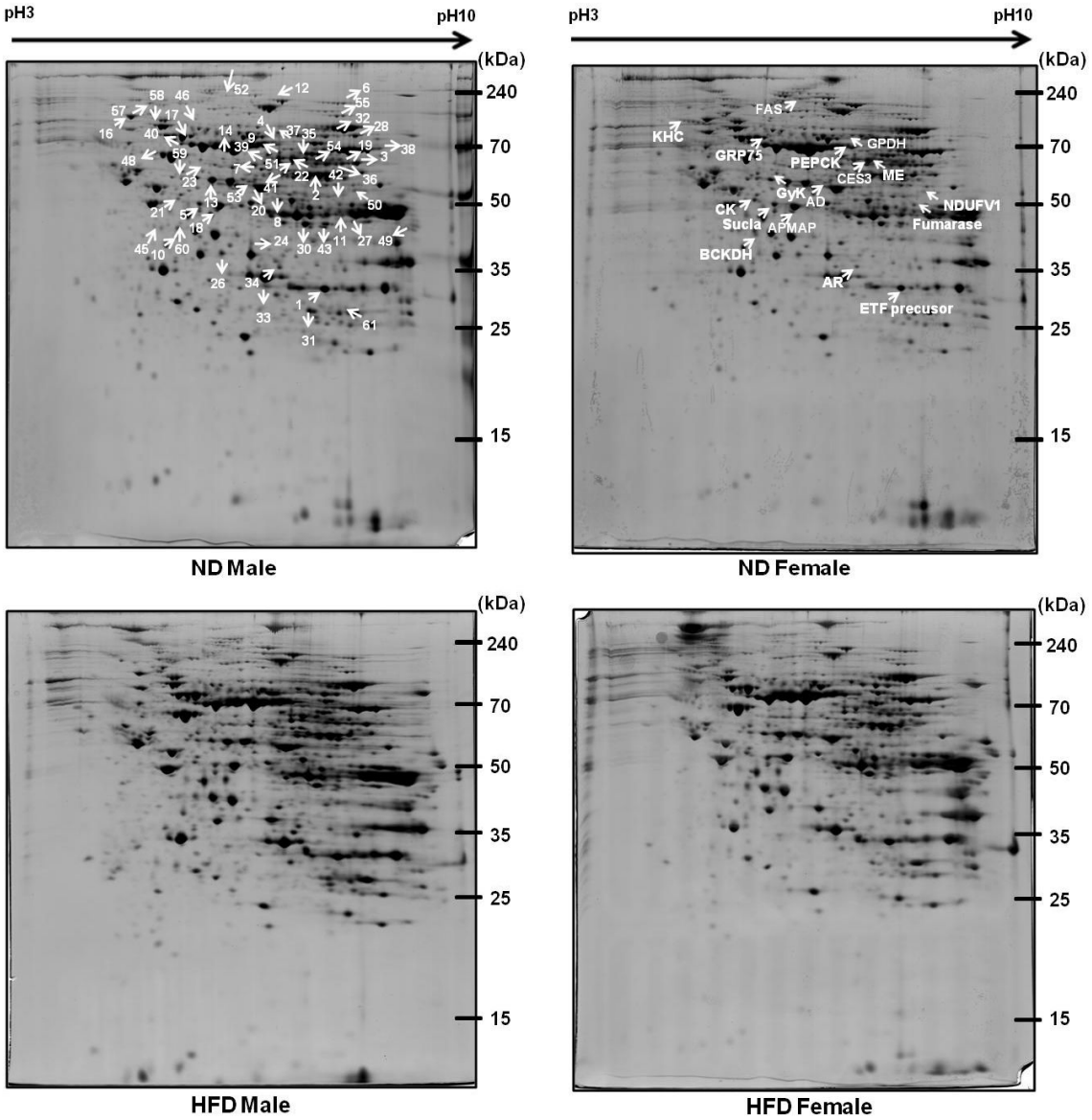

Fig. 2. Representative silver-stained 2-DE gel images of rat BAT proteome in ND and HFD rats. Differentially regulated proteins in each group are marked with arrows and proteins of numbers in gel are listed in Table 3.

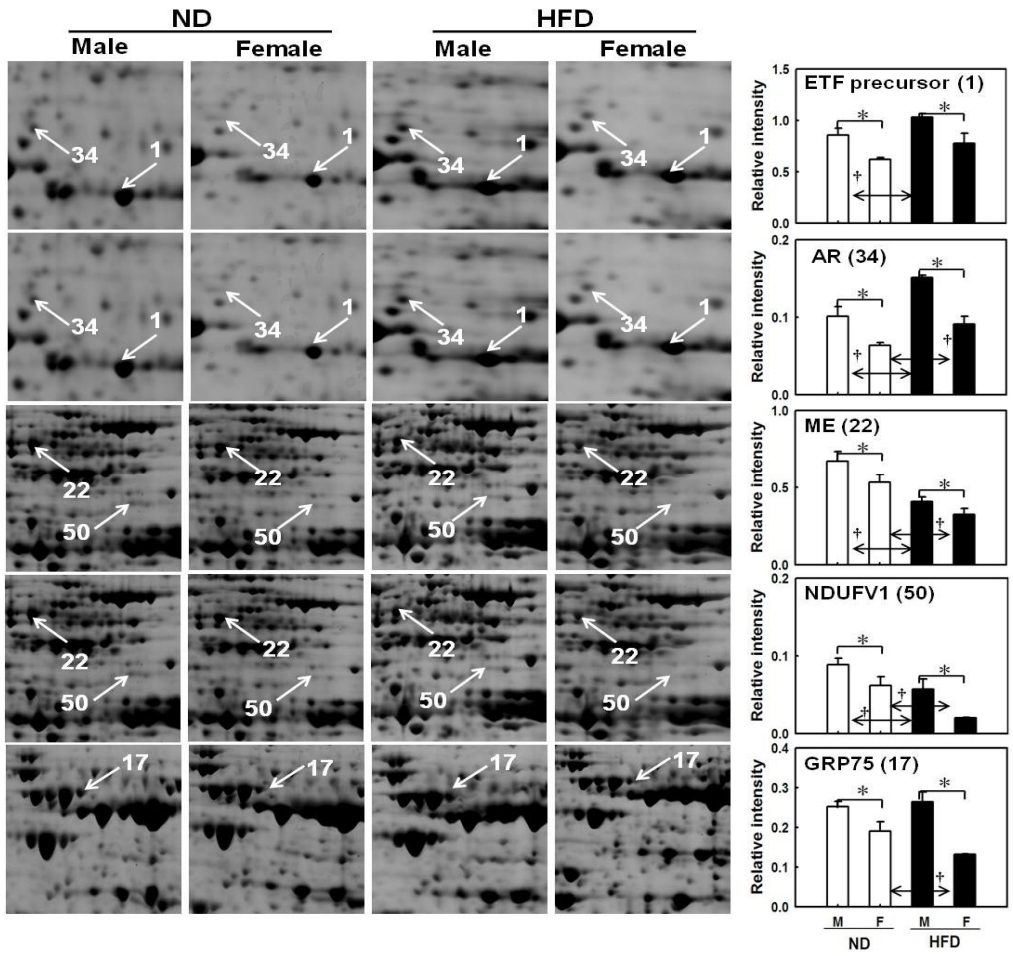

Fig. 3. Differentially expressed BAT proteins showing gender difference in both ND and HFD rats with identical patterns. Data are exhibited as mean values ±SEM of volume density (%) of the changed spot in three individual gels using pooled plasma of seven rats per group. These 12 proteins have p values below 0.05 between males and females fed ND and/or HFD. Statistical significance between male (M) and female (F) rats was determined by a t-test, where p values were $^{*}p<0.05$ and $^{**}p<0.01$and significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. For abbreviation of each protein name, see Table 3.

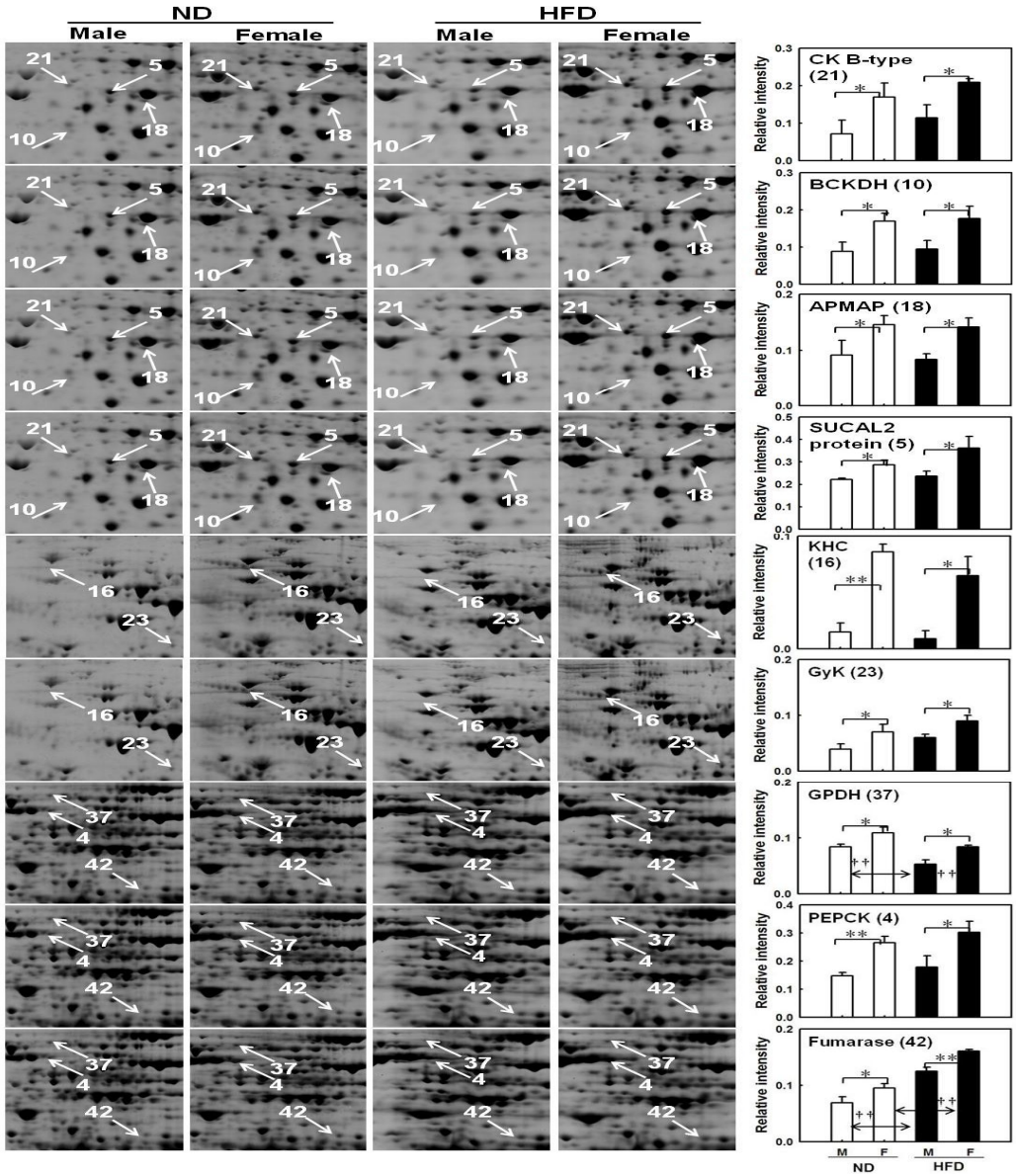

Fig. 4. Differentially expressed BAT proteins showing gender difference in both ND and HFD rats with opposite patterns. Data are exhibited as mean values ±SEM of volume density (%) of the changed spot in three individual gels using pooled plasma of seven rats per group. Statistical significance between male (M) and female (F) rats was determined by a t-test, where p values were $^{*}p<0.05$ and $^{**}p<0.01$ and significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. For abbreviation of each protein name, see Table 3.

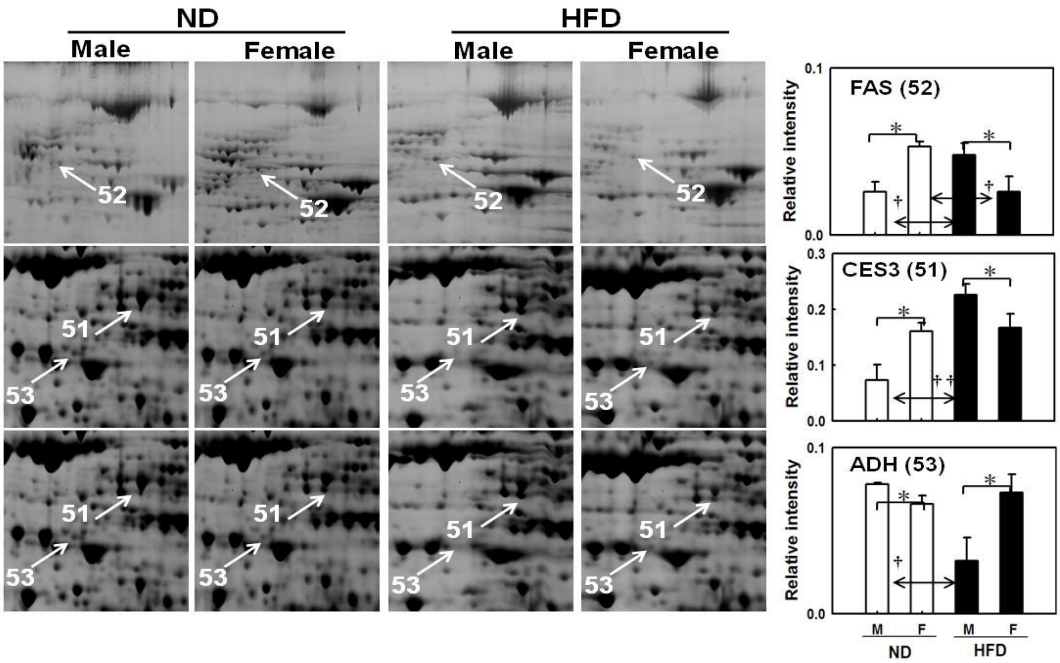

Fig. 5.Differentially expressed BAT proteins showing gender-difference in the regulation patterns in only ND or HFD (A) and no gender-difference but HFD response in both ND and HFD (B). Data are exhibited as mean values ±SEM of volume density (%) of the changed spot in three individual gels using pooled plasma of seven rats per group. Statistical significance between male (M) and female (F) rats was determined by a *t*-test, where *p* values were $^{*}p<0.05$ and $^{**}p<0.01$ and significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. For abbreviation of each protein name, see Table 2.

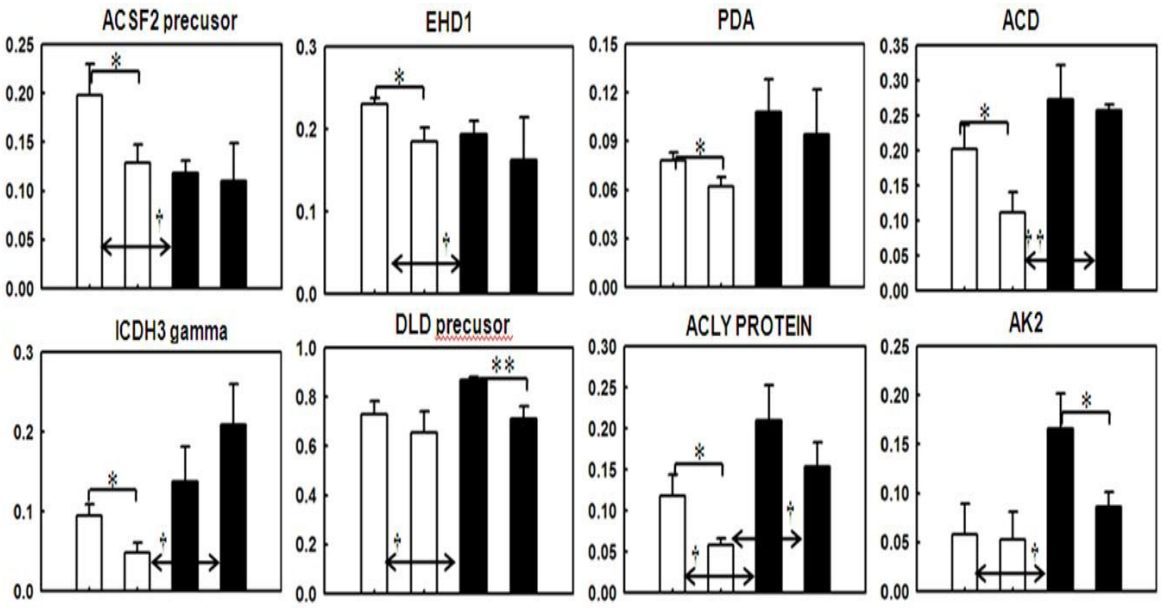

Fig. 6. Validation of differentially regulated BAT proteins in male (M) and female (F) rats in response to HFD by immunoblot analysis. Levels of 3 identified proteins from 2-DE analysis was established using pooled BAT samples of seven rats per group. Data are representative of three independent experiments. For abbreviation of each protein name, see abbreviations.

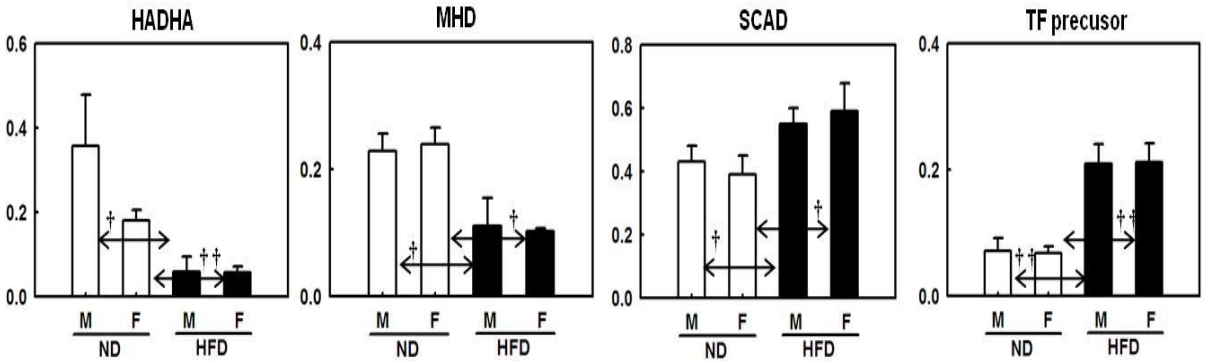

Fig. 7. Differentially regulated BAT proteins of metabolic importance in male (M) and female (F) rats in response to HFD by immunoblot analysis. Levels of 6 important BAT proteins were established using pooled BAT samples of seven rats per group. Data are representative of three independent experiments. For abbreviation of each protein name, see abbreviations

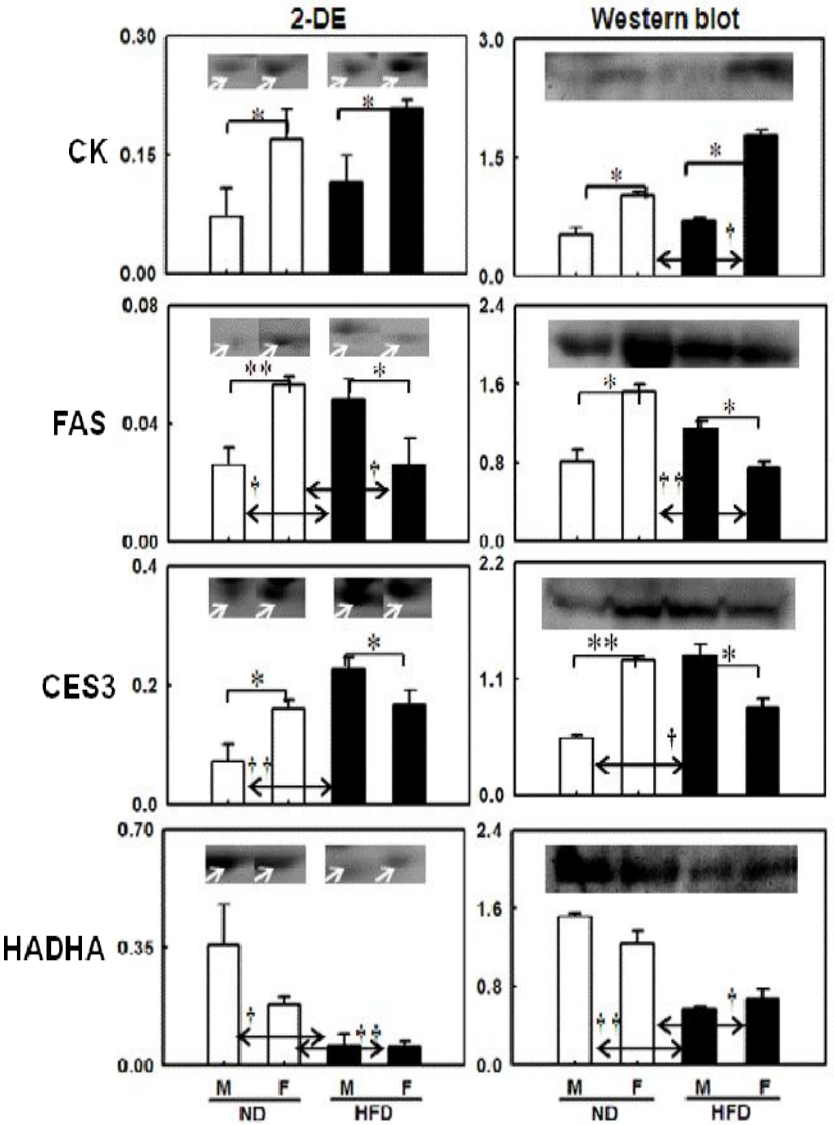

Fig. 8. Comparison of protein (A) and mRNA levels of UCP1 and (B) PGC1−α (C) as well as mitochondrial content (D) in male (M) and female (F) rats in response to HFD. All were established using pooled BAT samples of seven rats per group. Data are representative of three independent experiments. For abbreviation of each protein name, see abbreviations.

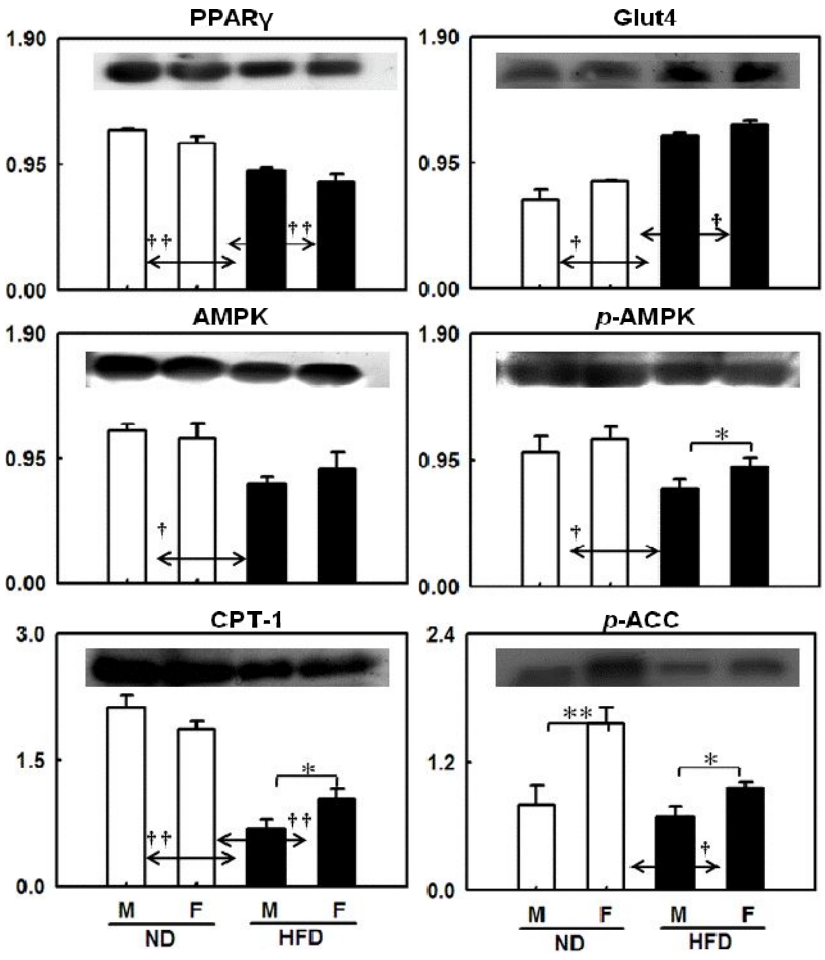

Fig. 9.Many protein showing a gender-different were associated with obesity and thermogenesis in BAT of female rats fed a high fat diet compared to those of male rats. Up-regulated proteins were marked in block and down- regulated proteins were marked white. Abbreviations: Fu, fumarase; OAA, oxaloacetate; A-KG, alpha-ketoglutarate. The remainder proteins see abbreviation section for the full name

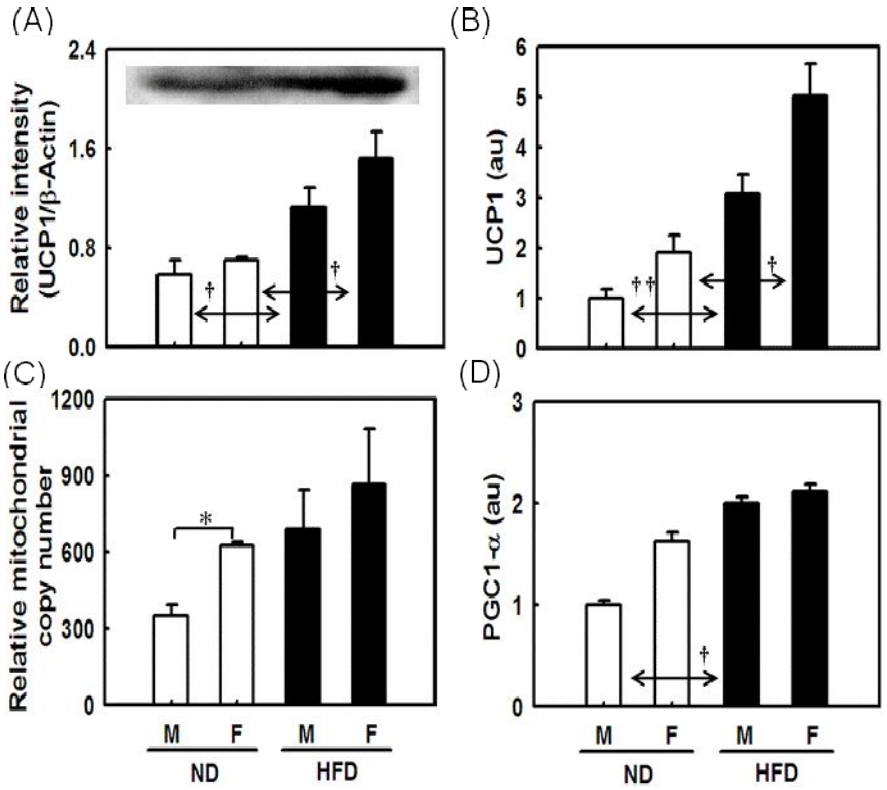

Fig 10. Comparison of protein (A) and mRNA levels of UCP1 (B), as well as PGC1-α (C) and mitochondrial content (D) in male (M) and female (F) rats in response to HFD. Protein levels were determined by Western blot analysis and mRNA levels were measured using real-time PCR. All were established using pooled BAT samples from 7 rats per group. Data are representative of 3 independent experiments. For abbreviation of each protein name, see abbreviations.

2. Discussion

In the present study, we explored comparative proteome analysis in BAT between male and female rats in response to HFD treatment with the hypothesis that members of the BAT proteins can be either directly or indirectly related to the control of body weight in both genders. A variety of studies have raised the possibility that there exist sex-associated differences in metabolic activity in adipose tissues such as energy expenditure and oxidative capacity (Rodríguez-Cuenca et al. 2002; Justo et al. 2005; Valle et al. 2007; Rodríguez et al., 2001). It is demonstrated that female rats show a greater oxidative capacity with more effective antioxidant machinery compared to males. The increased oxidative activity would result in higher energy intake but lower body fat deposition than males (Català-Niell et al. 2008). Compared to male rats, female rats have higher protein content (particularly UCP1), greater respiratory and phosphorylative capacities in mitochondria thereby resulting in higher energy expenditure (Rodríguez-Cuenca et al. 2002; Justo et al. 2005; Valle et al. 2007). Borrás and coworkes (2003) also found that females exhibited higher antioxidant gene expression and lower oxidative damage than males. Although they did not link their findings to obesity, this result also seems to be responsible to higher fat oxidation in females. During the postcafeteria period, BAT is more efficiently activated in the females who experienced higher body weight losses than males after removal of the cafeteria diet (Rodríguez et al., 2003). It is also demonstrated that female rats are more sensitive to cold than male rats and resisting cold is a physiological priority (Roca et al. 1999). Female rats are known to be more sensitive to cold because their threshold temperature for the thermogenesis response is set at a higher value (22°C) than that of males (18°C). Female rats might have a low threshold temperature for cold-induced thermogenic response. In other word, their BAT is already activated at 22°C,

whereas male rats are not (or less) sensitive to the cold at this temperature (Quevedo et al., 1998). Male and female animals acclimated to 22℃ has different specific UCP1 levels in BAT, which were double for female rats that for male rats (Quevedo et al., 1998).

However, several conflicting results suggesting higher thermogenic activity in males than females have been reported. For example, the higher levels of β_3-adrenoceptor in male rats than females have been demonstrated, pointing to greater thermogenic capacity and less weight gain in males (Rodríguez et al., 2001). In addition, female rats appeared to decrease energy expenditure by protecting metabolically active organs to a greater extent than male rats during calorie restriction (Valle et al. 2005). Rodríguez and Palou (2004) demonstrated a gender difference in cold, diet, and overweight-induced stimulation of the expression of the main uncoupling protein UCP1 in BAT. Upon chronic cafeteria diet feeding, female rats have been shown to attain a larger excess of body weight than males. BAT of female lean control rats has been shown to be already activated compared to that of males (Rodríguez and Palou 2004). Taken together, it remains to be determined whether males or females have higher innate thermogenic activity lead to suppress weight gain when exposed to HFD.

In the current study, we found numerous proteins showing differential expression in a gender-dependent manner at the 24 weeks of breeding duration. To interpret the result of proteomic analysis, we categorized identified proteins into three groups: (1) proteins showing a gender-difference in both ND and HFD rats (Fig 3), (2) proteins showing gender-difference both in ND or HFD rats with opposite patterns (Fig 4), and (3) Proteins showing a gender-difference in the regulation patterns in only ND or HFD rats (Fig 5A) and no gender-difference but HFD response (Fig 5B).. Due to space limitation, a limited number of proteins showing a clear gender difference were discussed here.

Proteins showing a gender-difference in the identical regulation patterns in both ND and HFD rats

Twenty three proteins are categorized into this group; 11 proteins showed higher expressions in males while 12 proteins marked higher levels in females. The first exciting finding in the current comparative proteomic study between the genders is profoundly higher expression levels of kinesin heavy chain (KHC, isoform 5C) in females (Fig. 3). Kinesin is a motor protein found in eukaryotic cells and its active movement supports several cellular functions including cell division and transport of cargo such as axonal transport (Vale et al. 1985; Kull et al. 1996). Krzysztof et al. 2007). Evidence has been accumulated in favor of the hypothesis that GLUT4 movements in adipocytes and muscle cells are dependent on cytoskeletal structure, and optimal GLUT4 translocation in response to insulin requires intact actin filaments (Wang et al. 1998; Omata et al. 2000). Semiz et al. (2003) found that kinesin mediates insulin-stimulated GLUT4 movements on adipocyte cell microtubules. Recent data also suggest that there is a connection between disruption of intraflagellar transport and obesity as evidenced by the discovery that proteins associated with human obesity (James et al. 2007). As will be discussed later, in this study, higher GLUT4 expression was observed in BAT of female rats (Fig. 7), suggesting that higher GLUT4 movement resulted in shifting glucose flux for TG accumulation. Therefore, due to striking differences in protein levels of kinesin between the genders, this protein can be used as a potential anorectic marker determining gender difference in BAT irrespective of diet condition.

We also found higher protein levels of creatine kinase (CK) in females (Fig. 3). In cold adaptation in BAT, the up-regulation of ATP synthase can be a response of the tissue in order to sustain the ATP availability needed for the housekeeping of the cold-adapted BAT. An event in BAT is the up-regulation of ATP synthase together with the down-regulation of CK. The ATP synthase

up-regulation point out the competition that exists between two proton electrochemical gradient-consuming systems, including one that is energy-conserving (ATP synthase), while the other one dissipates energy (UCP1). We believe that diet-induced thermogenesis in BAT may be the same case. Therefore, the higher levels of CK in female BAT suggest a greater capacity for using phosphocreatine as an energy buffer providing ATP for cytosolic reaction during uncoupling reaction in HFD-induced obese rats.

It is interesting to pay attention to higher levels of enzymes involved in glycerol pathway. Activation of BAT thermogenesis requires the hydrolysis of endogenous triacylglycerols to produce fatty acids (FA), which are both substrates and uncoupling messengers for BAT mitochondria (Festuccia et al. 2003). Therefore, maintenance of adequate stores of triacylglycerols, through esterification, via glycerol-3-phosphate (G3P), of newly synthesized or preformed FA seems to be essential for the normal functioning of BAT. One of the sources of G3P for acylation is the glycerol produced by hydrolysis of stored triacylglycerols or taken up by the tissue from the circulation that is directly phosphorylated to G3P by glycerolkinase. It has been demonstrated that in conditions of a sustained increase in BAT sympathetic flow there is a stimulation of glycerolkinase (GyK) gene expression at the pretranslational level, with increased enzyme activity, mediated by β-adrenoreceptors (Festuccia et al. 2003). Our finding supports this result in that higher protein levels of GyK together with phosphoenolpyruvate carboxylkinase (PEPCK) (Fig. 3) that is a regulatory enzyme of_glyceroneogenesis. We also found that higher of glycerol-3-phosphate dehydrogenase (GPDH) were observed in females (Fig. 3). However, our results argue strongly against the observations that overexpressions of GPDH, GyK or PEPCK led to diet-induced insulin resistance and obesity in white adipose or liver tissue (Franckhauser et al. 2006; MacDougald and Burant 2005). Taken together, role of these enzymes in BAT in determining fate of glycerol (maintenance of FFA and consequent uncoupling

messengers for BAT) is likely to be different from other tissues (TG accumulation using glycerol as a substrate). Therefore, results of gender-different expressions of GPDH, GyK or PEPCK point to a greater thermogenic activity in female rats.

One of the most striking results in our proteomic study is finding of different regulation patterns of two lipogenic proteins. The protein levels of malic enzyme (ME) (Fig. 3) and fatty acid synthase (FAS) (Fig. 4) in HFD-fed females were strikingly lower than males. Since ME activity positively correlates with rates of *de novo* fatty acid synthesis, lower ME levels point to relatively less fat accumulation in both ND- and HFD-fed female rats. Protein levels of FAS in BAT of male rats were higher in HFD rats than ND rats, but not in BAT of female rats. Since plasma lipid profiles are very similar between the genders, it is likely that the extent to which the excess lipid deposition in BAT of male rats may be not due to the uptake of triacylglycerol from plasma, but due to higher lipogenesis mediated by increased FAS levels within the tissue. In view of the apparent relationship between thermogenesis and lipogenesis in BAT it might be expected that fatty acid synthesis rates in the BAT of females would be low in obese rats. Collectively, it was consequently suggested that BAT might make a major contribution to the development of obesity in the male rats, by virtue of the export of fatty acids for storage in WAT.

Compared to males, down-regulation of aldose reductase (AR) in females is likely to contribute to favorable carbohydrate metabolism (Fig. 4). In response to the chronic hyperglycemia found in diabetics, glucose flux through the polyol pathway is significantly increased. Up to 33 % of total glucose utilization in some tissues can be through the polyol pathway. Glucose concentrations are often elevated in diabetics and AR has long been believed to be responsible for diabetic complications involving a number of organs.

We also found lower levels of females in the two proteins involved in the

mitochondrial respiratory chain, electron-transfer flavoprotein (ETF) and NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1) (Fig. 3). Although defects in these proteins are common cause of mitochondrial dysfunction, down-regulation of these proteins in females is likely to influence on enhanced uncoupling capacity in females.

We also detected higher levels of three different enzymes of the TCA cycle; (isocitrate dehydrogenase 3; Fig. 5, fumarase and Sucla2; Fig. 3) in female rats when fed a HFD , This greater substrate flux on the TCA cycle was likely to increase in β-oxidation enzymes in females suggesting more complete oxidation of fatty acids. Without increase in TCA cycle enzymes, higher β-oxidation enzymes would result in incomplete fat oxidation thereby impairing mitochondrial functions. Upregulation of branched-chain α-keto acid dehydrogenase complex (BCKADC) that is a combination of enzymes responsible for the degradation of the branched chain amino acids is also similar result, in that this complex is known to be analogous to the alpha-ketoglutarate dehydrogenase complex in the TCA cycle (Fig. 3).

Interestingly, higher levels were detected in adipocyte plasma membrane-associated protein (APMAP) in female BAT in both ND and HFD rats (Fig. 3). Although the precious biological function of APMAP is unknown at present, it is considered a marker of adipocyte differentiation, that is present mostly in mature adiposytes (Albrektsen et al. 2001). However, this was found only in WAT not in BAT. Taken together with the observation that female rats had higher BAT content than males, another clue for higher thermogenic activity in females could be derived from differential expression of APMAP between the genders when both were exposed to HFD.

The gender-dependent protein levels of GRP75 are another interesting finding in this study. GRP75 is a heat-shock cognate protein which is primarily localized to the mitochondria but is also existed in the endoplasmic reticulum (ER), plasma membrane and cytoplasmic vesicles. This protein plays a role in

cell proliferation, stress response and maintenance of the mitochondria (Ran et al. 2000). A protective mechanism used by cells to adapt to stress of the ER is the induction of members of the GRP family. The induction of mammalian GRP proteins in response to ER stress involves a complex network of regulators and novel mechanisms. The elucidation of GRP function and regulation opens up new therapeutic approaches to diseases associated with ER stress and cancer (Lee, 2001). It has been reported that pathological conditions such as tumor growth correlate with GRP overexpression (Little et al. 1994; Lee, 2001). Therefore, our findings raise the possibility that higher protein levels of GRP74 in males may reflect a more severe state of obesity.

Proteins showing gender-difference with opposite regulation patterns in ND and HFD rats.

HFD-responsive up-regulation of carboxylesterases 3(CES3) only in males is also interesting result in this study (Fig. 4). CESs are enzyme family that convert a carboxylic ester to an alcohol and a carboxylate, and has recently been suggested to play a role in lipolysis. Jernas and coworkers (Jernas et al. 2009) recently demonstrated that expression of the CES1 gene is highly regulated in human adipose tissue, with increased levels in obese subjects and decreased levels during weight loss. However, differential expression patterns of these enzymes in mammalian BAT have not been demonstrated. Our proteomic analysis of rat BAT pointed out that HFD-fed rats showed higher CES3 expression than ND-fed mice only in males, reflecting the sign of higher lipid accumulation in HFD-fed male rats but not in females.

Opposing regulation response of aldehyde dehydrogenase (ADH) to HFD between the genders is worthy to discuss (Fig. 4). In males, levels of ADH were markedly reduced in HFD rats, whereas no significant changes were observed in females. One possible explanation for this result could be the greater detoxifying role in lipid-derived oxidative stress because ADH was identified as a key

component of the detoxification pathway of aldehydes arising from lipid peroxidation events (Demozay et al. 2004).

The results of immunoblot analysis clearly supported the proteomic results, in that identical expression patterns for selected 3 proteins (e.g.CK, FAS, and HADHA). To confirm higher thermogenic activity in female BAT obtained from the proteomic study, we compared the mitochonderial content, levels of UCP1 (marker for energy expenditure) and PGC1-α (marker for mitochondrial biosynthesis and estrogen receptor activator) between the genders. Somewhat to our surprise, we found that expressions of all these showed gender-difference. In particularly, female rats exhibited significantly greater stimulation of UCP1 expression in both protein and mRNS levels than males.

In this study, we found interesting changes in the levels of estrogen and testosterone in response to HFD feeding. The levels of estrogen in both males and females were increased in HFD-fed rats. The higher magnitude of increase in females (30%) than males (15%) compared to ND rats was observed. In contrast, levels of testosterone in males were strikingly decreased (53%) but detectably increased in females (13%). Taken together, maintenance of high levels of estrogen also supports increased energy expenditure in HFD-fed female rats. Our finding is in line with the earlier results that mice of deficient estrogen receptor or aromatase accumulated higher fat than their littermates (Heine et al. 2000; Jones et al. 2000)

Current proteomic analysis of BAT in HFD-induced obesity allowed us to identify 48 proteins showing gender-specific regulation between male and female rats. Although some of these have already been linked to obesity, gender-dependent regulation patterns of most identified proteins are reported for the first time in this study. We found conclusive results that numerous proteins involved directly or indirectly in thermogenic action were greater expression in females. Moreover, traits of lower fat synthesis and higher oxidation activity in females were confirmed. We believe that gender-different

protein regulation in BAT was, at least in part, resulted from sex hormone. For example, estrogen might down regulate lipogenic enzymes and shifted partition of FFA toward oxidation away from TG storage by activations of AMPK. In conclusion, present proteomic research into gender-dimorphic pathophysiological mechanisms would aid in improvement of gender awareness in the health care system and in implementation of evidence-based gender specific clinical recommendations.

Chapter 2. Gender-dependent gene expressions in brown adipose tissue of lean and obese rats fed a high fat diet.

1. Results

1.1. HFD-induced phenotypes in male and female rats

Male and female rats were randomly separated into 2 groups, with 20 rats fed an ND and 40 rats fed an HFD, respectively. When changed to the experimental diets, body weight and food intake of ND and HFD rats were almost identical at the beginning in both male and female rats. However, after 2 weeks, body weights in HFD-fed rats were significantly higher ($p<0.05$) than those in their ND-fed counterparts at all subsequent time points in both male and female rats (Fig. 1A), while body weight gain were higher in female HFD rats by an average of about 38% than that of the LFD rats ($p=0.07\text{x}10^{-3;}$ Fig. 1A, right). Total body weight gain per food intake of HFD-fed male rats was higher than in females by an average of ~30% (p = 0.001) (Fig. 1B). These phenotypic differences prompted us to perform further genomic studies.

1.2 Global microarray data analysis

Oligonucleotide microarray analyses were conducted in order to determine any changes in the gene expression profile of BAT between both genders. Expression profiling was performed for BAT from normal and HFD rats ($n=3$). In each group, the gene expressions were generated using the Illumina RatRef-12 Bead Chip, which includes 22,523 probe sets representing >13,186 known genes and 8,030 expressed sequence tags with unknown functions. In this study, the PANTHER classification system (http://www.pantherdb.org) was used for classification of genes according to their functions (Kim et al., 2008). In brief, gene lists can be uploaded to the size and analyzed relative to molecular functions, biological processes, and pathways.

The majority of genes showing significant differences between males and

females were genes encoding proteins/enzymes of carbohydrate and lipid metabolism, apoptosis, regulation of vasoconstriction, cell cycle control, cell structure, coenzyme metabolism, oxidative phosphorylation, ectoderm development, mesoderm development, other homeostasis activity, T-cell mediated immunity, mRNA transcription, purine metabolism, other carbon metabolism, protein modification, translation regulation, cell surface receptor mediated signal transduction, intracellular signaling cascade, as well as extracellular transport and import (Tables 1 and 2). This screening process led to identification of 55 genes that were found to have significant changes in expression of the BAT, as depicted in the dendrograms (Fig. 2). Furthermore, we compared gene expression profiles by the SAM two-class unpaired test method with criteria of two fold or higher between the groups. A hierarchical clustering of genes that were differentially regulated in BAT of rats fed a ND and HFD for 8 weeks is shown in Fig. 2. In addition to genes associated with lipid and carbohydrate metabolism, genes involved in apoptosis, cell cycle control, cell structure, coenzyme metabolism, signal transduction were overrepresented (also see Table 2).

To view global changes in genes in response to an HFD and between male and female rats, we constructed two different scatter plots based on the total 22523 genes: ND rats vs. HFD rats for each gender (Fig. 3A) as well as males vs. females for each diet (Fig. 3B). To this end, we extracted and counted the numbers of those genes up- or down-regulated more than 2-fold between the genders and diets. Using this complementary approach, we were able to detect genes at a glance showing differential expressions of 2-fold or higher between genders and diets. Scatter plot analysis revealed that a greater number of genes of BAT in females showed dramatically higher differential expression with higher magnitude, compared with those of males (Fig. 3A right and Fig. 3B right).

1.3. Differential expression of genes involved in lipid metabolism and thermognic

regulation

Next, other two scatter plots were made again for in-depth view of the gene changes involved in lipid metabolism and thermognic regulation (Fig. 4). Interestingly, greater number of genes involved in fatty acid oxidation indicated higher expression in females than males (female genes 29 vs. male genes 13) when exposed to HFD (Fig. 4A). Similarly, expression levels of thermogenic genes such as Ucp1 and Cox also marked higher numbers in females than males (female genes 23 vs. male genes 7) after HFD feeding (Fig. 4B).

1.4. Validation of microarray data by real-time PCR

Four selected differentially expressed genes identified (e.g. *Acox1*, *Cpt1b*, *Fabp3*, *Gpd2*, and *Pygm*) on the microarray were validated by quantitative real-time PCR. As shown in Fig. 5, all five genes displayed an identical expression patterns on both diets and genders as established by microarray analysis.

Table 4. Sequence of primers used for real-time PCR.

Gene symbol	Gene description	Forward	Reverse
Acox1	acyl-Coenzyme A oxidase	5'-CACGCAATAGTTCTGGCTCA-3'	5'-ACCTGGGCGTATTTCATCAG-3
Cpt1b	carnitine palmitoyltransferase 1b	5'-GCAAACTGGACCGAGAAGAG-3'	5'-CCTTGAAGAAGCGACCTTTG-3'
Fabp3	Fatty acid binding protein3	5'-ACGTTGCCTCATGTTTCTCC-3	5'-GCCTTGGCTCTGCTTTATTG-3'
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	5'- AGACAGCCGCATCTTCTTGT-3'	5'-CTTGCCGTGGGTAGAGTCAT-3'
Gpd2	glycerol-3-phosphate dehydrogenase 2,	5'-TGGGCTTGAGAGTGAGGTTGC-3'	5'-GCCACCGTTTTCCAGTCACA-3'
Pygm	Glycogen phosphorylase	5'-CTAGCTGCCTATGGCTACGG-3'	5'-GCTCCACTCGACCGTAGAAG-3'

Table 5. Differentially expressed (2-fold change) genes in brown adipose tissue (BAT) of male (M) and female (F) fed either normal diet (ND) or high fat diet (HFD), identified through microarray profiling.

Gene symbol	Gene description	Accession No.	ND-F/ ND-M[a)]	HFD-F/ HFD-M[a)]	HFD-M/ ND-M[a)]	HFD-F/ ND-F[a)]
Apoptosis						
Maged1	melanoma antigen, family D, 1	NM_053409.1	**2.17**	-1.45	1.97	-1.61
Sgk	serum/glucocorticoid regulated kinase	NM_019232.1	**-2.97**	-1.03	**-3.40**	-1.18
Regulation of vasoconstriction, dilation						
Aplnr	Rattus norvegicus apelin receptor (Aplnr), mRNA.	NM_031349.2	**-2.05**	-1.25	-1.39	1.17
Glycogen metabolism						
Gys2	glycogen synthase 2	NM_013089.1	**2.45**	1.86	1.29	-1.02
Pygm	glycogen phosphorylase	XM_342002.3	-1.57	**2.27**	-1.69	**2.11**
Cell cycle control						
Fos	FBJ murine osteosarcoma viral oncogene homolog.	NM_022197.1	-1.72	1.36	**-2.30**	1.02
Jun	Jun oncogene	NM_021835.3	**-2.00**	-1.01	-1.71	1.16
Nedd9	neural precursor cell expressed, developmentally down-regulated 9	NM_001011922.1	**-2.08**	-1.17	-1.77	1.00
Dusp1	dual specificity phosphatase 1	NM_053769.2	**-2.29**	1.44	**-2.01**	1.65
Junb	jun B proto-oncogene	NM_021836.2	**-2.69**	1.15	**-2.62**	1.18
Btg2	B-cell translocation gene 2, anti-proliferative	NM_017259.1	**-3.10**	-1.09	**-3.26**	-1.15
Cyr61	cysteine-rich, angiogenic inducer, 61	NM_031327.2	**-3.44**	1.03	**-3.64**	-1.03
Cell structure						
Myot	myotilin	XM_214563.4	-1.41	**2.12**	-1.32	**2.26**
Actn3	actinin alpha 3.	NM_133424.1	-1.82	**2.67**	-1.84	**2.64**
Col3a1	collagen, type III, alpha 1	NM_032085.1	-1.89	-1.18	**-2.00**	-1.26
Myh11	myosin, heavy polypeptide 11	XM_573030.2	**-2.07**	1.15	-1.73	1.38
Actg2	actin, gamma 2	NM_012893.1	**-2.14**	1.62	-1.81	1.91
Tpm1	tropomyosin 1, alpha	NM_001034075.1	**-2.41**	**2.80**	**-2.33**	**2.89**
Acta1	actin, alpha 1	NM_019212.2	**-4.33**	**5.89**	**-4.24**	**6.01**
Cldn5	claudin 5	NM_031701.2	**-4.82**	-1.08	**-5.08**	-1.13
Coenzyme metabolism						
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	NM_017268.1	**2.27**	-1.00	**2.48**	1.09
Pank1	pantothenate kinase 1 (predicted) (Pank1_predicted), mRNA.	XM_215283.4	**2.24**	1.16	1.62	-1.20
Peci	peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase	NM_001006966.1	**2.12**	1.35	1.38	-1.14
Oxidative phosphorylation						
Cabc1	chaperone, ABC1 activity of bc1 complex homolog	NM_001013185.1	-1.27	**-2.47**	-1.32	**-2.57**
Ectoderm development						
Ulk1	unc-51-like kinase 1	XM_001080656.1	-1.83	1.02	**-2.11**	-1.14
Adora2a	adenosine A2a receptor	NM_053294.3	**-2.02**	1.21	-1.71	1.44
Egr2	early growth response 2	NM_053633.1	**-2.48**	1.04	**-3.15**	-1.22

(Continued)

Mesoderm development						
Nfatc4	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4.	XM_001061815.1	**2.05**	-1.01	1.52	-1.36
Ttn	titin (Ttn), mRNA.	XM_001065955.1	**-2.20**	**3.28**	**-2.17**	**3.31**
Dusp1	dual specificity phosphatase 1	NM_053769.2	**-2.29**	1.44	**-2.01**	1.65
Cyr61	cysteine-rich, angiogenic inducer, 61	NM_031327.2	**-3.44**	1.03	**-3.64**	-1.03
Other homeostasis activities						
Aplnr	apelin receptor	NM_031349.2	**-2.05**	-1.25	-1.39	1.17
Aqp1	aquaporin 1	NM_012778.1	**-2.22**	-1.00	**-1.54**	1.44
T-cell mediated immunity						
RT1-CE7	RT1 class I, CE7.	NM_001008845.1	**-2.01**	-1.24	-1.63	1.00
mRNA transcription						
Klf2	Kruppel-like factor 2	NM_001007684.1	**-2.40**	-1.23	**-2.48**	-1.27
Egr1	early growth response 1	NM_012551.1	**-3.94**	1.08	**-3.08**	1.38
Purine metabolism						
Gmpr	guanosine monophosphate reductase	NM_057188.1	**4.34**	1.79	**2.05**	-1.18
Other carbon metabolism						
Car4	carbonic anhydrase 4	NM_019174.1	**-2.19**	1.26	**-2.03**	1.36
Protein modification						
Prkar2b	protein kinase, cAMP dependent regulatory, type II beta	XM_343046.3	**2.12**	1.08	1.72	-1.14
Dusp1	dual specificity phosphatase 1	NM_053769.2	**-2.29**	1.44	**-2.01**	1.65
Snf1lk	SNF1-like kinase.	NM_021693.1	**-2.66**	-1.10	**-3.39**	-1.40
Translational regulation						
Eef1a2	eukaryotic translation elongation factor 1 alpha 2.	NM_012660.2	**-1.74**	**2.04**	-1.66	**2.13**
Cell surface receptor mediated signal transduction						
Pdgfrl	platelet-derived growth factor receptor-like	NM_001011921.1	-1.59	-1.28	**-2.05**	-1.65
Rgs2	regulator of G-protein signaling 2	NM_053453.1	**-2.01**	**-3.30**	-1.20	**-1.97**
Aplnr	apelin receptor	NM_031349.2	**-2.05**	-1.25	-1.39	1.17
Rgs3	regulator of G-protein signaling 3	NM_019340.1	**-3.62**	1.39	**-3.46**	1.45
Intracellular signaling cascade						
Tnnc2	troponin C type 2	NM_001037351.1	**-2.12**	**3.19**	**-2.31**	**2.94**
Zfp36	zinc finger protein 36	NM_133290.2	**-2.45**	-1.02	-1.78	1.35
Extracellular transport and import						
Abcb9	ATP-binding cassette, sub-family B (MDR/TAP), member 9	NM_022238.1	1.79	1.07	**2.13**	1.27
Slc22a3	solute carrier family 22 (extraneuronal monoamine transporter), member 3	NM_019230.1	1.08	-1.64	-1.32	**-2.34**
Miscellaneous						
Ckm	creatine kinase	NM_012530.1	**-2.36**	**3.15**	**-2.26**	**3.29**
Ankrd15	ankyrin repeat domain 15	XM_001055725.1	**-2.77**	-1.17	**-2.51**	-1.06
Ier2	immediate early response 2	NM_001009541.1	**-2.63**	1.16	**-2.55**	1.19
LOC290704	similar to palladin	XM_214338.3	**-2.42**	-1.18	**-1.96**	1.05
Fam82a	family with sequence similarity 82	NM_001037200.1	**2.22**	1.32	**1.54**	-1.09

[a] Differentially expressed genes between the genders and diets are represented, where the fold change ratios are the average fold change using the SAM two class unpaired test method.

Table 6. Differentially expressed genes associated with lipid metabolism in brown adipose tissue (BAT) of male (M) and female (F) fed either normal diet (ND) or high fat diet (HFD), identified through microarray profiling.

Gene symbol	Gene description	Accession No.	ND-F/ ND-M[a)]	HFD-F/ HFD-M[a)]	HFD-M/ ND-M[a)]	HFD-F/ ND-F[a)]
Fatty acid metabolism						
Peci	peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase	NM_001006966.1	**2.12**	1.35	1.38	-1.14
Decr2	2,4-dienoyl CoA reductase 2, peroxisomal	NM_171996.2	**2.05**	1.24	1.48	-1.12
Cyp2e1	cytochrome P450, family 2, subfamily e, polypeptide 1	NM_031543.1	**2.02**	-1.31	1.36	**-1.95**
Acsl1	acyl-CoA synthetase long-chain family member 1	NM_012820.1	**1.89**	1.06	1.49	-1.19
Crat	carnitine acetyltransferase	NM_001004085.2	**1.62**	1.10	1.38	-1.07
Ech1	enoyl coenzyme A hydratase 1, peroxisomal.	NM_022594.1	**1.61**	-1.05	1.40	-1.21
Acsl5	acyl-CoA synthetase long-chain family member 5	NM_053607.1	**1.54**	1.15	-1.24	**-1.67**
Acox1	acyl-Coenzyme A oxidase 1, palmitoyl	NM_017340.1	**1.54**	1.10	1.30	-1.08
Hadh	hydroxyacyl-Coenzyme A dehydrogenase	NM_057186.1	**1.53**	1.12	1.30	-1.05
Cpt1b	carnitine palmitoyltransferase 1b	NM_013200.1	**1.51**	1.27	1.42	1.20
Slc27a1	solute carrier family 27 (fatty acid transporter), member 1	NM_053580.2	1.19	1.00	-1.34	**-1.59**
Echdc1	enoyl Coenzyme A hydratase domain containing 1	NM_001007734.1	-1.01	**-1.53**	1.02	-1.48
Acss2	PREDICTED: acyl-CoA synthetase short-chain family member 2	XM_230773.4	-1.21	**-1.75**	-1.26	**-1.82**
Aacs	acetoacetyl-CoA synthetase	NM_023104.1	-1.31	1.03	**-1.54**	-1.15
Lipid metabolism						
Neu2	neuraminidase 2 (Neu2), mRNA.	NM_017130.1	**1.74**	1.31	**1.75**	1.33
Gpd2	glycerol-3-phosphate dehydrogenase 2,	NM_012736.1	**1.61**	1.17	1.42	1.03
Ugdh	UDP-glucose dehydrogenase	NM_031325.1	-1.07	-1.01	**-1.56**	-1.47
Fdft1	farnesyl diphosphate farnesyl transferase 1.	NM_019238.2	-1.34	**-1.54**	-1.41	**-1.62**
St3gal2	ST3 beta-galactoside alpha-2,3-sialyltransferase 2.	NM_031695.1	**-1.53**	-1.20	-1.21	1.06
Rnpep	arginyl aminopeptidase .	NM_031097.1	**-1.71**	-1.43	**-1.54**	-1.29
Amacr	alpha-methylacyl-CoA racemase	NM_012816.1	1.47	-1.07	**1.55**	-1.02
Agpat2	PREDICTED:1-acylglycerol-3-phosphate O-acyltransferase 2	XM_001074707.1	**1.89**	-1.09	**1.78**	-1.16
Chkb	choline kinase beta .	NM_017177.1	**1.87**	1.10	**1.57**	-1.08
Gpd1	glycerol-3-phosphate dehydrogenase 1	NM_022215.2	**1.60**	1.21	1.41	1.06
Crls1	cardiolipin synthase 1	NM_001014258.1	1.34	-1.29	1.13	**-1.53**

Lipid and fatty acid binding						
Fabp3	fatty acid binding protein 3	NM_024162.1	**5.12**	**2.53**	**3.09**	**1.53**
Abcd3	ATP-binding cassette, sub-family D	NM_012804.1	1.44	-1.10	**1.66**	1.05
Sorl1	PREDICTED: sortilin-related receptor, L(DLR class) A repeats-containing	XM_001065506.1	1.29	-1.10	**1.60**	1.13
Slc27a1	solute carrier family 27	NM_053580.2	1.19	1.00	-1.34	**-1.59**
Tmem176b	transmembrane protein 176B .	NM_134390.2	**-1.51**	-1.04	-1.36	1.07
Regulation of lipid, fatty acid and steroid metabolism						
LOC501283	PREDICTED: similar to lipid droplet associated protein	XM_001061544.1	**1.76**	-1.08	**2.28**	1.20
Rxrg	retinoid X receptor gamma	NM_031765.1	-1.42	**-1.79**	-1.39	**-1.74**
Steroid metabolism						
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	NM_017268.1	**2.27**	-1.00	**2.48**	1.09
Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1.	NM_017286.1	**2.01**	**1.68**	1.38	1.15
Dgat1	diacylglycerol O-acyltransferase homolog 1 .	NM_053437.1	**1.58**	1.07	1.29	-1.14

) Differentially expressed genes between the genders and diets are represented, where the fold change ratios are the average fold change using the SAM two class unpaired test method

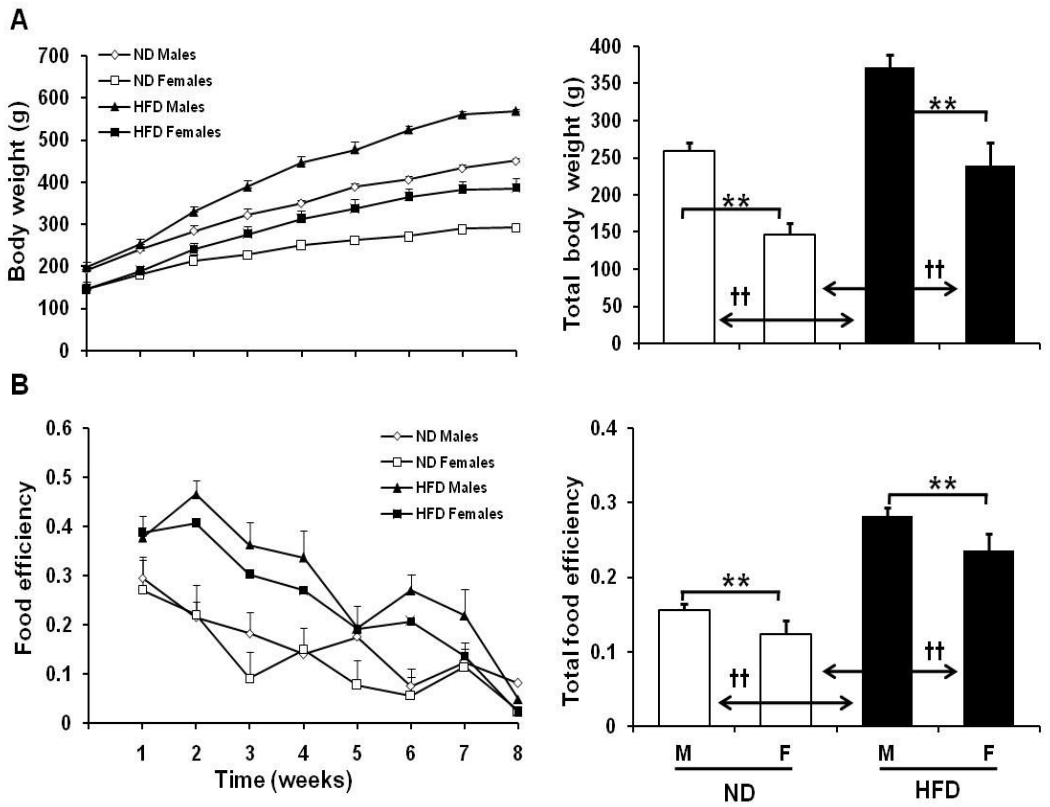

Fig. 11. Body weight profile (A) and food efficiency (B) in male and female rats fed ND and HFD. Data are presented as mean ± SEM for 7 rats per group and were estimated using the ANOVA test. Statistical significance between male and female rats was determined by a t−test, where p value is $^{*}p<0.05$ and $^{**}p<0.01$, and significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$.

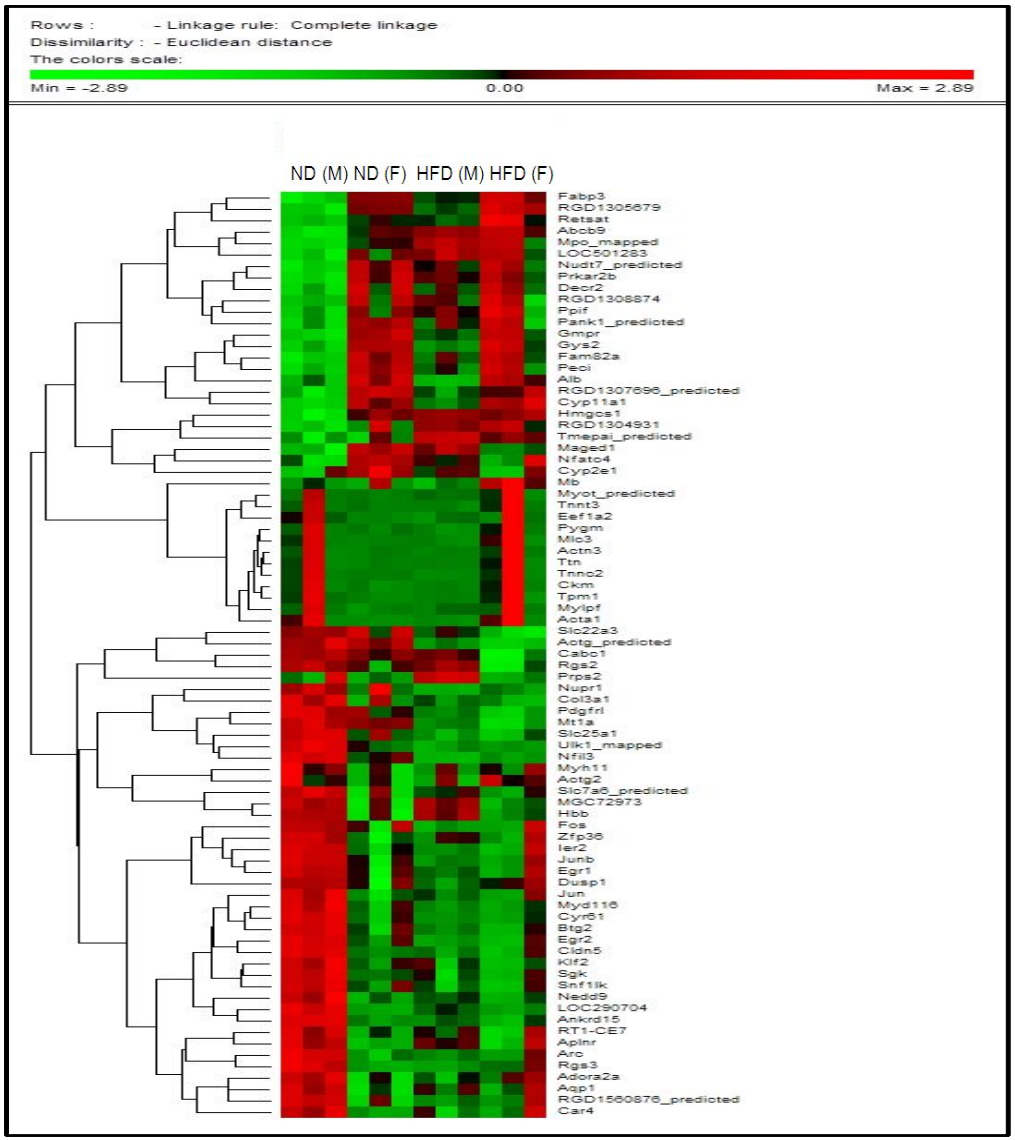

Fig. 12. Hierarchical clustering of BAT genes in male (M) and female (F) rats before (ND) and after (HFD) HFD feeding. All of genes in the Illumina chip were clustered based on their expression patterns across 12 samples using R 2.4.1 software 2.4.1 (www.r-project.org). Expression level of representation is used to a green to red color palette, relative to the mean expression level of the control group, green (down-expression), red (up-expression) and blank (missed or excluded date).

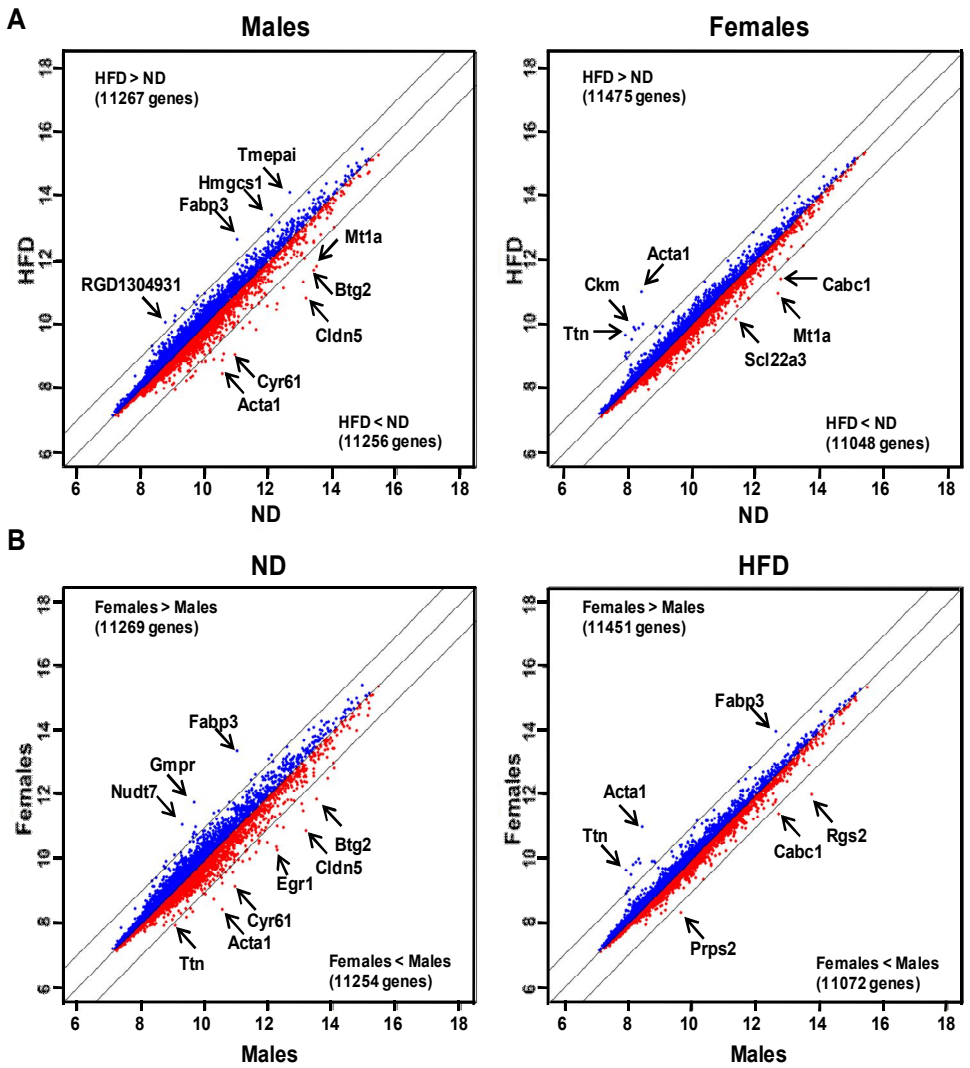

Fig. 13. Global comparison of gene expressions in BAT from male and female rats. Scatter plots of the signal intensities of individual genes for rats fed a normal (ND) or high fat diet (HFD). Blue (up-regulated) and red (down-regulated) dots indicate genes. In each graph, individual genes were plotted on the x- and y-axis to show the signal intensities observed for rats fed ND or HFD, respectively. Upper and lower diagonal lines display the two-fold regression lines, respectively. Transcript expression levels are on the $\log_2$ scale.

For abbreviation of each gene. Abbreviations: *Acta*, actin alpha 1; *Btg2*, b-cell translocation gene 2; *Cabc1*, Chaperone activity of bc1 complex-like *Ck*, creatine kinase; *Cldn5*, claudin 5; *Cyr61*, cysteine-rich; *Egr1*, early growth response 1; *Fabp3*, fatty acid binding protein; *Gmpr*, guanosine monophosphate reductase; *Hmgcs*, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1; *Mt1a*, metallothionein 1a; *Nudt7*, Nucleoside diphosphate-linked moiety X motif 7; *Prps2*, phosphoribosyl pyrophosphate synthetase 2; *Rgd1304931*, RIKEN cDNA 2310042D19; *Rgs2*, regulator of G-protein signaling 2; *Scl25a1*, solute carrier family 25 (mitochondrial carrier, citrate transporter); *Tmepai*, transmembrane, prostate androgen induced RNA; *Ttn*, titin

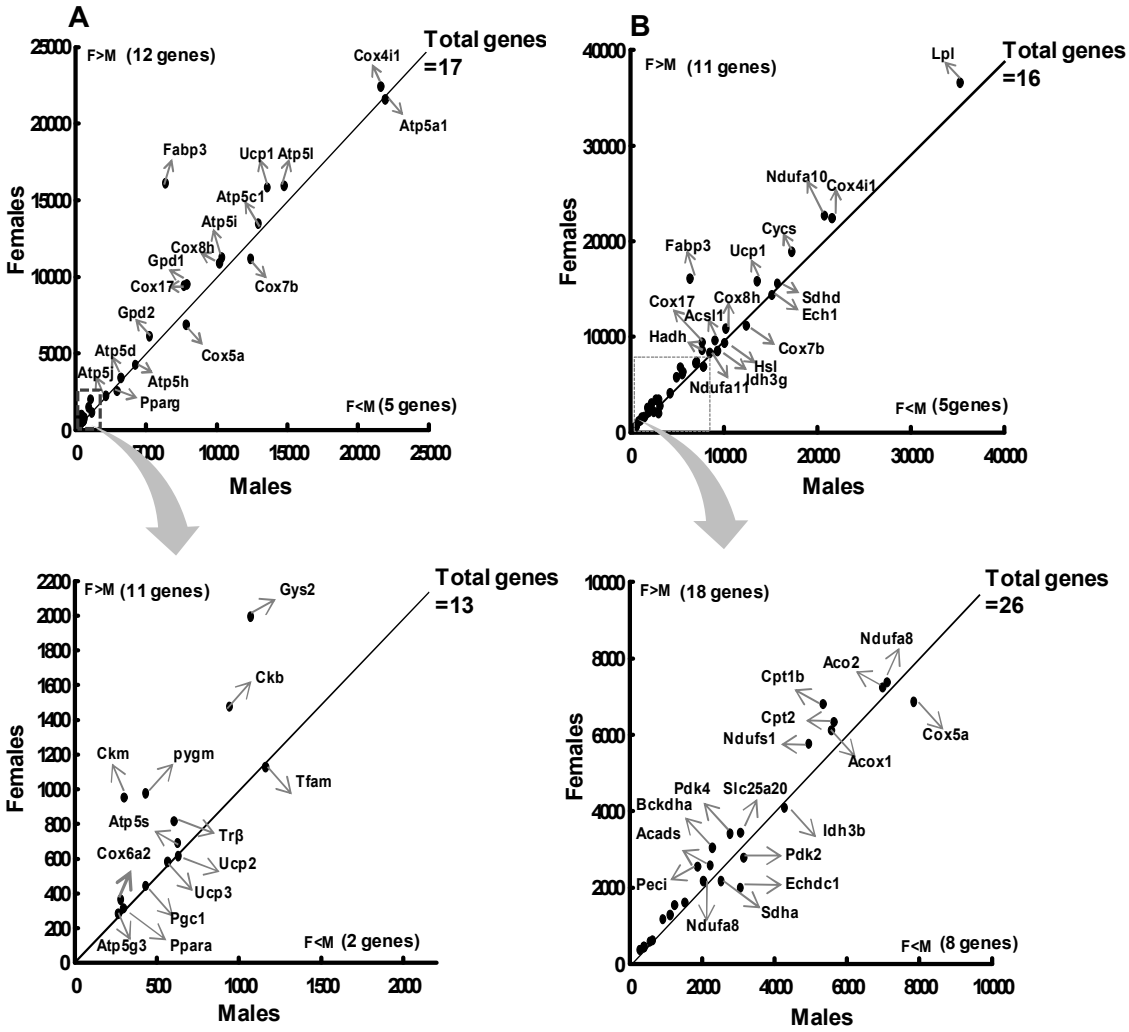

Fig. 14. Scatter plots comparing differential expression of genes in BAT of HFD rats involved in thermognic regulation (A) and fat oxidation (B). Scatter plots of the signal intensities of individual genes for rats fed a normal (ND) or high fat diet (HFD). In each graph, individual genes were plotted on the x− and y−axis to show the signal intensities observed for rats fed ND or HFD, respectively. Abbreviations: *Acad*, acyl−Coenzyme A dehydrogenase; *Aco*, aconitase ; *Acsl*, acyl−coa synthetase long−chain; *Acox*, acyl−coenzyme a oxidase; *Atp*, ATP synthease; *Bckdh*, branched chain keto acid dehydrogenase; *Ck*, creatine kinase; *Cox*, cytochrome C oxidase; *Cpt*, carnitine palmitoyltransferase; *Cycs*,

cytochrome c; *Decr*, 2,4-dienoyl coa reductase; *Ech*, enoyl coenzyme A hydratase; *Fabp*, fatty acid binding protein ; *Gpd*, glycerol-3-phosphate dehydrogenase; *Gys*, glycogen synthase; *Hadh*, hydroxyacyl-coenzyme dehydrogenase; *Hsl*, hormone sensitive lipase; *Idh*, isocitrate; *Lpl*, lipoprotein lipase; *Nduf*, NADH dehydrogenase (ubiquinone) flavoprotein; *Pdk*, pyruvate dehydrogenase kinase; *Pdp*, pyruvate dehydrogenase phosphatase; *Peci*, delta2-enoyl-coenzyme a; *Pgc*, peroxisome proliferator-activated receptor gamma coactivator; *Ppar*, peroxisome proliferator-activated receptors; *Pygm*, glycogen phosphorylase; *Sdha*, succinate dehydrogenase; *Slc*, solute carrier (carnitine/acylcarnitine translocase); *Tfam*, transcription factor A, mitochondrial; *Tr*β, thyroid hormone receptor; *Ucp*; uncoupling protein

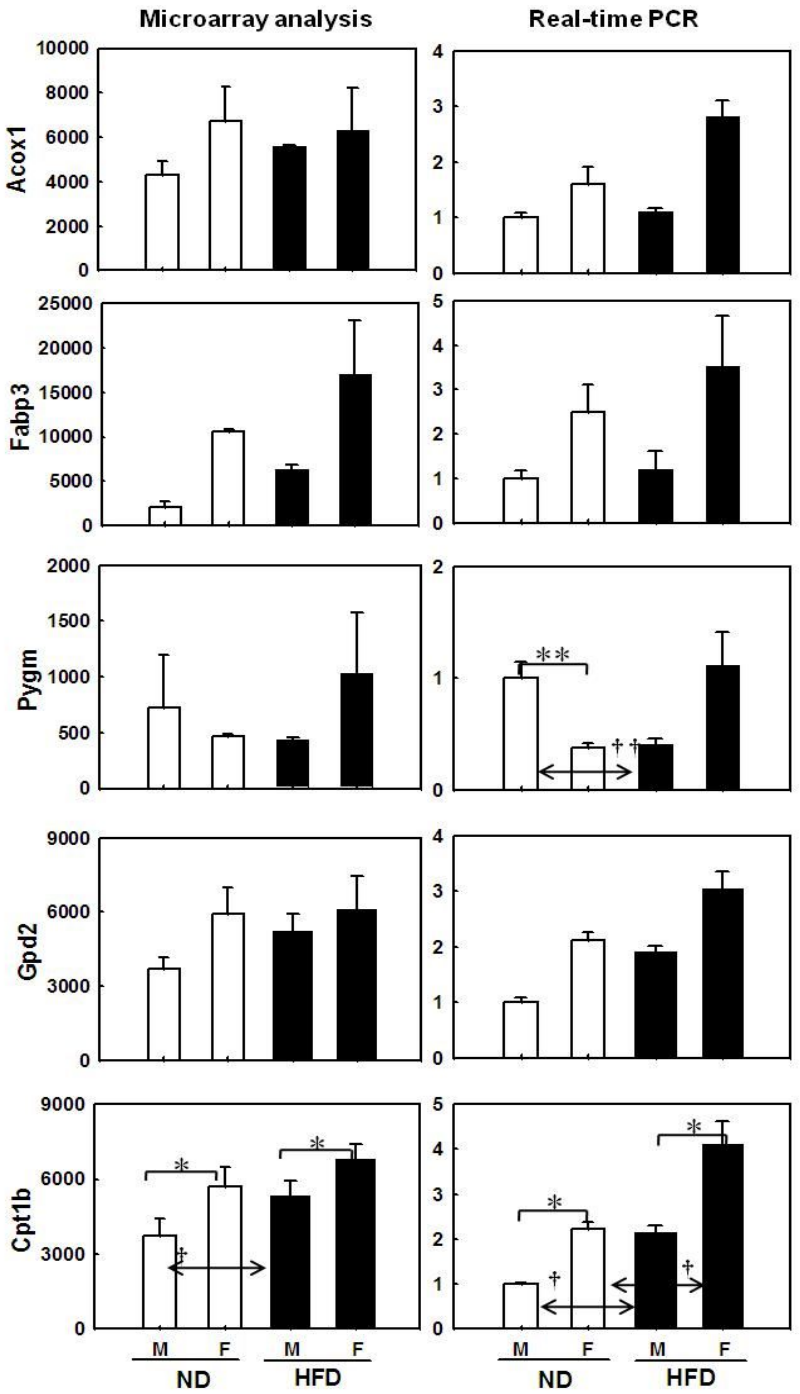

Fig. 15. Validation of microarray data (left) by comparing with real-time PCR data (right) for five important genes. Levels of five genes were normalized to those of *Gapdh* gene expression and represent as the mean ± SD of triplicate

independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ for significance, and significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. Abbreviations: *Acox*, acyl-coenzyme a oxidase; *Cpt*, carnitine palmitoyltransferase; *Fabp*, fatty acid binding protein; *Gpd*, glycerol-3-phosphate dehydrogenase; *Pygm*, glycogen phosphorylase

2. Discussion

In the present study, based on the hypothesis that expressions of members of the BAT genes can be either directly or indirectly related to control of body weight in both genders, we explored comparative gene expression analysis in BAT between male and female rats before and after HFD feeding.

A variety of studies have raised the possibility for the existence of sex-associated differences in metabolic activity in adipose tissues, such as energy expenditure and oxidative capacity (Justo et al., 2005; Rodriguez-Cuenca et al., 2002; Rodriguez et al., 2001; Valle et al., 2007). A growing body of evidence indicates that female rats show a greater oxidative capacity with more effective antioxidant machinery compared to males (Borras et al., 2003; Justo et al., 2005; Rodriguez-Cuenca et al., 2002; Valle et al., 2007). Compared to male rats, female rats have higher mitochondrial protein content (particularly UCP1), and greater respiratory and phosphorylative capacities in mitochondria, leading to higher energy expenditure (Justo et al., 2005; Rodriguez-Cuenca et al., 2002; Valle et al., 2007). The increased oxidative activity would result in higher energy intake but lower body fat deposition, compared with males (Catala-Niell et al., 2008).

However, several conflicting results suggesting higher thermogenic activity in males than females have also been reported (Rodriguez and Palou, 2004a; Valle et al., 2005). For example, higher thermogenic activity in females is restricted to only non-hyperphagic condition. Conversely, in a situation of high adrenergic stimulation, as occurs with overfeeding, male rats have a greater thermogenic activity than female rats through higher levels of β_3-adrenoceptor (Rodriguez et al., 2001). In addition, female rats appeared to have decreased energy expenditure through protection of metabolically active organs to a greater extent than male rats during calorie restriction (Valle et al., 2005). Rodríguez and Palou (2004) demonstrated a gender differences in cold, diet, and overweight-induced stimulation of expression of UCP1 in BAT. Upon chronic

cafeteria diet feeding, female rats have been shown to attain a larger excess of body weight than males. BAT of female lean control rats has been shown to be already activated, compared to that of male (Rodriguez and Palou, 2004a).

Taken together, it remains to be determined whether males or females have higher innate thermogenic activity leading to suppression of weight gain when exposed to HFD. Therefore, present microarray study was aimed to seek the answers to numerous questions about gender-different gene regulation as well as molecular abnormalities of diet-induced obesity. Consequently, we were able to discover a total of 55 genes (2-fold change or higher changes) associated with lipid metabolism, as well as 35 genes (1.5-fold change or higher changes) involved in thermogenic regulation between males and females.

It is well recognized that reinforcement of fatty acid oxidation and energy expenditure in BAT has been reported to decrease obese, fat deposition and body weight gain (Rosen and Spiegelman, 2006). Therefore, we focused on differential expression of genes associated with lipid metabolism and energy expenditure. We found that significantly regulated genes in beta-oxidation were up-regulated in both ND and HFD rats in female rats, including fatty acid binding protein 3 (*Fabp3*), acyl-CoA synthetase long-chan family member1 (*Acsl1*), carnitine palmitoyltransferase 1b,2 (*Cpt1b,2*), 2,4- dienoyl CoA reductase 2 (*Decr2*), acyl-coenzyme A oxidase 1(*Acox1*), hydroxyacyl-coenzyme dehydrogenase (*Hadh*), delta2-enoyl-coenzyme A isomerase (*Peci*), and lipoprotein liapse (*Lpl*) (see Table 2).

Especially, *Fabp3* was the most up-regulated in both ND and HFD female rats. *Fabp3* is_ a gene involved in active fatty acid metabolism where it transports fatty acids from the cell membrane to mitochondria for oxidation (Kleine et al., 1992). *Fabp3* expression is known to be correlated with UCP1 function and the degree of heat requirement. A similar observation on the cold-induced expression of *Fabp3* mRNA in BAT was previously reported by Yamashita et al., 2008. Considering the primary function of Fabp3, its marked increase in BAT

would supply a large amount of long-chain fatty acids for the mitochondria, where Ucp1 efficiently "burns" the substrate to make heat in the cold condition (Vergnes et al., 2011; Yamashita et al., 2008). Therefore, higher expression of *Fabp3* in females suggests that higher fat oxidation is directly or indirectly linked to thermogenic activity in females.

Because free fatty acids (FFAs) are endogenous energy sources in adipose tissue, increasing the relative percentage of FFA oxidation could be an effective means to decrease triglyceride (TG) stores and achieve weight loss. Thus, understanding the role of key regulatory enzymes of fat partitioning is essential for modulating the control of FFA oxidation (Zang et al., 2005).

Among many genes associated with beta-oxidation, *Cpt1* is an essential gene as it encodes protein transports long-chain fatty acids from outer mitochondrial membrane to inner mitochondrial membrane by binding them to carnitine (McGarry et al., 1989). Meanwhile, *Cpt2* encodes a protein transporting long-chain fatty acids from inner mitochondrial membrane to mitochondrial matrix for beta-oxidation. *Acsls* is as essential gene encoding membrane ligase family in pre-step reaction for beta-oxidation such that mediates fatty acid degradation by catalyszing ATP-dependent acylation of fatty acids into long-chain acyl CoAs (LCA-CoAs), thereby entering the β-oxidation pathway for energy production or undergo further esterification for production of phospholipids, cholesterol esters, and triglycerides (Parkes et al., 2006). Similarly, *Decr2* encodes an accessory enzyme which participates in the beta oxidation and metabolism of unsaturated fatty enoyl-CoA esters. Higher expression of *Lpl* in females also support greater fat oxidative capacity of female rats. Collectively, most of genes encoding enzymes associated with fatty acid oxidation were showed obviously higher in females than males in BAT (Fig. 4B), suggesting that these differences might cause significant changes in total fat deposition and fatty acid degradation in BAT.

Expectedly, majority of thermogenic genes such as genes encoding uncoupling

proteins (particularly Ucp1) and gene families encoding ATP synthase in BAT also showed gender-different expressions albeit their magnitudes were less than those of fat oxidative genes. Examples included genes encoding creatine kinase (*Ckm*), ATP synthease (*Atp*), glycogen synthase (*Gys*), glycogen phosphorylase (*Pygm*), and cytochrome C oxidase (*Cox*). Among these genes, *Ckm* is closely associated with energy metabolism, catalyses using ATP for the conversion of creatine to produce phosphocreatine and ADP. Interestingly, *Ckm* expression patterns were opposite between ND and HFD. Consequently, higher levels of female HFD rats but not female ND rats, suggest greater capacity for use of phosphocreatine as an energy buffer providing ATP higher in obese female rats than in males during thermogenesis (Choi et al., 2011).

It is noteworthy to discuss about higher expressions of *Atp* gene families and *Ckm* in females when fed a HFD. An earlier study demonstrated that in cold adaptation, the up-regulation of ATP synthase could be a response of the cell in order to sustain the ATP availability needed for the house-keeping of the cold-adapted brown adipocytes (Navet et al., 2007). The up-regulation of ATP synthase points out the competition that exists between two proton electrochemical gradient-consuming systems, including one that is energy-conserving (ATP synthase), while the other one dissipates energy (UCP1). Higher levels of *Ck* in female BAT suggest a greater capacity for use of phosphocreatine as an energy buffer providing ATP for cytosolic reactions during uncoupling reactions in HFD-induced obese rats.

We also observed significant difference in expression of genes in glucose and glycerol metabolisms in BAT between males and females, including glycogen synthase (*Gys2*), glycogen phosphorylase (*Pygm*) and glycerol-3-phosphate dehydrogenase (*Gpd*) (Tables 2, 3 and Fig. 5). Glycogen and glucose as well as hydrolysis of endogenous triacylglycerols for fatty acid are important fuels for thermogenesis because these are used for BAT mitochondrial substrates and uncoupling messengers (Festuccia et al., 2003; Himms-Hagen, 1990; Trayhurn,

1995). Activation of BAT for thermogenesis requires hydrolysis of endogenous triacylglycerols for production of fatty acids (FA), which are both substrates and uncoupling messengers for BAT mitochondria (Festuccia et al., 2003).

Gys and *Pygm* are key genes encoding enzymes for glycogen metabolism, where Gys catalyzes conversion of short glucose polymers into long polymers for storage as glycogen, whereas Pygm catalyzes the decomposition of glycogen to release glucose−1−phosphate (Azpiazu et al., 2000). Accordingly, we believe that glycogen and glucose−1−production production through interaction of *Gys2* and *Pygm* consequently influence on thermogenesis of female rats. This enabled the BAT to take up the glucose from the blood and utilize it directly for thermogenesis, or to store it as glycogen for later use (Madar and Harel, 1991). Higher expression of *Gpd* in females is also support this postulation because it is involved in maintaining the redox potential across the inner mitochondrial membrane glycolysis as well as adjust energy metabolism through forming the glycerol phosphate cycle (Harding et al., 1975).

Thyroid hormone plays a fundamental role in obligatory and adaptive thermogenesis by accelerating ATP turnover and expenditure. The Gpd activity is increased by thyroid hormone and this stimulation has been proposed to contribute to the increased metabolic rate caused by thyroid hormone (Gong et al., 1998; Lee and Lardy, 1965).

Activation of BAT for thermogenesis requires hydrolysis of endogenous triacylglycerols for production of fatty acids, which are both substrates and uncoupling messengers for BAT mitochondria (Festuccia et al., 2003). Therefore, maintenance of adequate stores of triacylglycerols, through esterification of newly synthesized or preformed FFA *via* glycerol−3−phosphate (G3P), appears to be essential for normal functioning of BAT. We also observed higher gene expression levels of *Gpd* in female rats than males, reflecting a link to greater thermogenesis and energy metabolism in females.

In conclusion, we report herein for the first time that a series of genes which

might be involved in fatty acid oxidation and thermogenic regulation were highly expressed in females than males, reaching the following two solid conclusions. First, compared to males females have greater fat clearing capacity through activation of genes encoding enzymes of fat oxidation. Second, females have higher thermogenic capacity via greater gene expressions involved in energy expenditure. Present microarray data of gender dimorphism in BAT would improve gender awareness in the health care system and to implement evidence-based gender specific clinical recommendations.

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쥐의 갈색지방조직에서 바이오마커 발굴을 위한 단백질체 분석과 유전체 분석

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(국문 초록)

1. 일반 식이와 고지방 식이를 섭취한 암컷 쥐와 수컷 쥐의 갈색지방 조직에서 비만 관련 단백질 바이오마커 연구.

본 연구에서는 8 주간에 일반 식이와 고지방 식이를 섭취한 암컷과 수컷 쥐의 갈색지방 조직에서 비만 관련 바이오 마커를 발견하여 남녀간의 비만에 대한 민간성과 매커니즘에 대하여 연구 하였다. 약 8 주간의 일반식이와 고지방식이를 섭취한 암컷과 수컷 쥐의 몸무게 결과, 수컷 쥐에게서 많은 양의 체중이 증가 한 것을 관찰 할 수 있었다.

따라서 비만 관련 바이오마커 발굴을 위한 쥐의 갈색지방조직의 단백질을 2 차원 전기영동을 분석한 결과, 총 48 개의 발현량 차이를 보이는 단백질을 발견 하였으며, 발현패턴에 따라서 다섯 그룹으로 나뉘었다. 2 차원 전기 영동 결과 암컷 쥐에게서 많은 양의 thermogenesis activity 와 지방 산화가 발생하였으며, 적은 양의 지방이 축적 된 것을 확인 할 수 있었다.

그리고 갈색지방조직에서 에너지 소비와 관련된 단백질들 (PPARγ, Glut4, p-ACC, AMPK, p-AMPK, CPT1, UCP1)의 발현량을 비교 분석한 결과, 암컷 쥐에게서 수컷 쥐보다 많은 양의 에너지 소비와 지방 산화가 활발하게 진행되고 있는 것을 확인하였다.

결론적으로 암컷 쥐 보다 수컷 쥐가 비만에 더욱 민간하며, 본 연구는 앞으로 가까운 미래에 남녀간의 특이적인 비만 치료제나 의약품 개발 및 발전에 크게 기여 할 것이다.

2. Microarray assay 을 통한 암컷 쥐와 수컷 쥐의 갈색지방조직에서 비만 관련 유전체 연구.

같은 일반 식이와 고지방 식이를 섭취 하여도 암컷 쥐와 수컷 쥐 간에 비만이 유도 되는 현상을 다르게 나타난다. 이러한 차이를 설명하기 위하여 우리는 8 주간의 일반식이와 고지방 식이를 섭취한 암컷 쥐와 수컷 쥐의 갈색지방조직에서 microarray assay 통한 유전체 분석을 실시 하였다.

유전체 분석 결과 갈색지방조직에서 발현량 차이 (2-fold change)가 큰 55 개의 유전자와 에너지 대사에 관련된 35 개의 유전자를 발견하여 유전자들의 기능에 따라서 각각의 그룹으로 나누었다. 그리고 발현된 많은 유전자들을 지방산화와 에너지 소비 기능에 따라 두 그룹으로 나뉘었다.

결과적으로 암컷 쥐에게서 수컷 쥐 보다 많은 양의 에너지가 소모 (thermogenesis activity)가 일어 났으며, 많은 양의 지방이 산화 되었다는 것을 관찰 할 수 있었다. 그리고 유전체 분석의 발현 차이를 확인 하기 위하여 real-time RT PCR 을 통해 확인 하였으며, 확인한 결과 microarray assay 분석 결과와 발현 패턴이 일치 하는 것을 관찰 할 수 있었다. 이 밖에도 암컷과 수컷간의 비만 관련 유전자들의 큰 발현차이를 확인 할 수 있었다.

결론적으로 암컷의 쥐에게서 수컷 쥐 보다 많은 양의 에너지가 소모되었으며, 많은 양의 지방이 산화 된 결과 암컷 쥐에게서 적은 양의 체중이 증가 하였다. 본 연구의 이와 같은 유전자 발현 패턴 연구를 통해 남녀 간의 비만에 특이적인 유전자 치료제 및 의약품이 개발에 큰 도움이 될 것 이라 생각 된다.

감사의 글

제 인생에 있어서 대구대학교 석사과정은 중요한 전환점이 되었습니다. 많이 부족하고, 큰 능력이 없는 나를 2 년동안 진심으로 이끌어 주시고, 논문을 완성 할 수 있도록 연구에 대한 열정과 자부심을 심어 주셨으며, 학문에 대한 열정과 연구자의 자세를 가르켜 주신 윤종원 지도 교수님의 큰 은혜를 평생토록 잊지 못 할 것 같습니다. 그리고 학과에 대한 한결 같은 애정으로 학부와 대학원 과정 동안 많은 가르침을 주셨던 송치헌 교수님, 문혜연 교수님, 박흠대 교수님, 강선철 교수님, 구덕본 교수님에게 진심으로 감사에 말씀을 드립니다.

2 년동안 힘든 실험 속에서 항상 도움과 많은 조언을 해 주셨던 최정원 박사님께 감사 드립니다. 힘든 실험실 생활 속에서 서로에게 힘이 되었던 우리 실험실 식구들 오태석, 라집 무케르지, 왕협, 류호 에게도 감사 드립니다. 나보다 1 년 일찍 졸업 하였지만, 실험실 생활에 큰 힘이 되었던 주정인 선배와 동기인 김동현과 다른 실험실 이지만 항상 많은 도움을 주었던 민성훈 선배, 박진모 선배, 이창환 선배, 손명주에게서 감사의 말을 전합니다.

마지막으로 저에게 있어서 평생 동안 못 갚을 사랑과 은혜를 주시며 저의 든든한 버팀목이 되어주신 소중한 아버지와 어머니 그리고 하나뿐인 누나에게도 큰 감사에 말씀을 드립니다.

이 감사의 글을 마무리하며 제가 혼자서 살아온 것이 아니고 여러분들의 도움을 받으며 지금까지 살라온 것을 다시 한번 되새기게 되었고, 이 고마운 분들에게 감사하다는 말 밖에 할 수 없는 것이 너무 죄송스러울 따름입니다. 이 많은 분들에게 부끄럽지 않도록 앞으로 더욱 부지런 하고 열심히 사는 모습을 보여 드리겠습니다.

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최덕권