Original Paper

The Effect of Growth Hormone on Lipid Accumulation or Maturation in Adipocytes

Yuchao Zhang^{a,b,c} Hongsheng Yu^d Peng Gao^a Jicui Chen^a Cong Yu^a Chen Zong^a
Sumei Lu^e Xia Li^a Xiaoli Ma^b Yuantao Liu^b Xiangdong Wang^{a,f}

^aDepartment of Cell Biology, Shandong University School of Medicine, Jinan, ^bDepartment of Endocrinology, Qingdao Municipal Hospital, Qingdao, ^cCenter for Reproductive Medicine, Provincial Hospital Affiliated to Shandong University, Jinan, ^dThe Department of Clinical Laboratory, the Affiliated Hospital of Ningbo University School of Medicine, Ningbo, ^eDepartment of Laboratory Medicine, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan, Shandong, ^fKey Laboratory of Protein Sciences for Chronic Degenerative Diseases in Universities of Shandong (Shandong University), Jinan, China

Key Words

Growth hormone (GH) • 3T3-F442A adipocyte • GH intervention • Peroxisome proliferator-activated receptor γ (PPAR γ) • Uncoupling protein-1 (UCP1)

Abstract

Background: Adipogenesis of adipocytes includes two stages: initiation and maturation. Growth hormone (GH) secretion is decreased in obese subjects and GH levels are inversely correlated with abdominal fat mass. The effects of growth hormone (GH) on lipids accumulation or maturation of adipocytes remains elusive. **Methods:** In the present study, effect of GH on lipid accumulation *in vitro* and *in vivo* was examined. cDNA microarray, quantitative real time-PCR (qPCR) and western blotting was used to analyze the expression of genes related to adipocyte lipid accumulation or degradation in pre- or mature 3T3-F442A adipocytes treated with GH and in epididymal adipose tissue of C57BL/6 mice administrated with GH. Level of adiponectin in supernatants of cultured F442A adipocytes was determined by enzyme-linked immune-sorbent assay. **Results:** We found that in 3T3-F442A especially 6 days post initiation of adipogenesis, GH intervention resulted in decreased expression of adipocyte maturation regulators (*C/EBP α* , *PPAR γ*) and prominent genes related to lipid synthesis such as *FAS* and *FABP*, while the expression of *UCP1* was markedly enhanced. cDNA microarray analysis and qPCR showed that the expression of *SOCS2* and *Adipor2* was increased under GH-treatment in mature 3T3-F442A adipocytes. GH treatment increased the mRNA expression of *adiponectin* and *UCP1* in mature adipocytes. The above results were confirmed by *in vivo* study. **Conclusions:** GH potentially negatively modulates the maturation and accumulation of lipid in adipocytes.

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Xiangdong Wang, PhD
and Yuantao Liu, MD and PhD

Department of Cell Biology, Shandong University School of Medicine, 44 Wenhua Road West, Jinan, 250012 (China) and Department of Endocrinology, Qingdao Municipal Hospital, Qingdao, 266071 (China); E-Mail xdongw@sdu.edu.cn/sdliuyuantao@163.com

Introduction

Growth hormone (GH), a 22-kDa polypeptide, is secreted in anterior pituitary gland, exerts important biological and physiologic effects [1, 2]. Pulsatile GH secretion plays a major role in controlling longitudinal growth in children, and in the human body, GH stimulates protein synthesis at the whole-body level, whereas in muscle tissue it inhibits glucose disposal [3, 4]. Growth hormone has also been shown to be involved adiposity. In obese subjects, GH secretion is reduced, and GH levels are inversely correlated with abdominal fat mass; GH secretion can be restored after a massive weight loss [5].

GH exerts biological effects directly on target cells by binding to cell membrane receptors (GHR) or/and through insulin-like growth hormone factor-I (IGF-I). The binding of GH to GHR activates the tyrosine kinase Janus kinase 2 (JAK2) and triggers a series of signal pathways including the signal phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 5 (STAT5) pathway [6]. GH activates PI3K and ERK signaling pathways similar to that of insulin which is an essential hormone for adipogenesis, therefore, GH may have similar effects on the initiation and transformation of pre-adipocyte differentiation into adipocyte, as suggested by Doglio and Nixon [7, 8]. Our previous data showed that insulin has a synergistic effect on GH induced STAT5 activation [9].

Obesity is a chronic metabolic disease due to fat accumulation [10], and is associated with increased risks of vascular diseases, inflammation, metabolic syndrome and type II diabetes [11, 12]. Obesity can be considered as a hypertrophic disease resulted from an increase in the number or size of individual adipocytes. Adipocytes are differentiated from pre-adipocytes. Under certain conditions, GH acts on adipose tissue to increase lipolysis, leading to the release of free fatty acid (FFA) into the blood in human and rodents [6]. In addition, treatment with GH in patients with growth hormone deficiency decreased fat mass and leptin levels [13]. GH stimulated lipolysis has been demonstrated in animal study [14]. However, GH was also shown to promote adipose differentiation in other studies [7, 8]. Therefore, the precise effect of GH on the fate of adipocytes needs further investigation. The related mechanism of GH modulating the size of adipocyte or the mass of adipose tissue also needs exploring.

In the present study, we designed a serial of experiments to determine whether GH inhibits adipocyte maturation or lipid accumulation in transformed pre-adipocyte during adipogenesis.

Materials and Methods

Reagents and antibodies

Cell culture medium and fetal bovine serum (FBS) were from Hyclone (ThermoScientific, Inc., Illinois, USA). Recombinant human GH was kindly provided by Eli Lilly & Co. (Indianapolis, IN). Primers used in this study were synthesized in Genomics Institute of HuaDa in Beijing. All other reagents were purchased from Sigma (St. Louis, MO, USA). Polyclonal antibody against CCAAT/enhancer binding protein α (C/EBP α), peroxisome proliferator-activated receptor γ (PPAR γ) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA); polyclonal antibody against fatty acid synthase (FAS) was from Proteintech Group, Inc (Wuhan, China); monoclonal antibody against α -tubulin was from Sigma (St. Louis, MO, USA). All secondary antibodies were purchased from ZhongShanJinQiao, Inc (Beijing, China).

Culture and differentiation of 3T3-F442A pre-adipocytes

The mouse embryo fibroblasts 3T3-F442A were cultured and induced to differentiate as described previously [9]. In brief, cells were cultured in Dulbecco's modified Eagle's medium containing (DMEM) 4.5g/L glucose, supplemented with 10% FBS, 50 μ g/mL streptomycin and 100 U/mL penicillin until they reached confluence. To induce differentiation, confluent cells were cultured in DMEM containing 10% FBS, 10 μ M insulin, 1 μ M dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. Medium was changed after 2 days with DMEM containing 10% FBS and 10 μ M insulin. Cells were then cultures with DMEM containing

10% FBS every other day. At 12 days, approximately 80% 3T3-F442A cells were differentiated into adipocytes as confirmed by Oil Red-O staining.

GH treatment in mature adipocytes or GH intervention during the process of adipogenesis

To investigate the role of GH in the process of adipocyte differentiation, 3T3-F442A pre-adipocytes were treated with GH (125 ng/mL) during the whole process of adipogenesis. In order to clarify the role of GH in mature adipocytes, differentiated 3T3-F442A cells were treated with GH (at final concentration of 125 ng/mL). Cells without GH treatment in both experiments were used as controls.

Animal experiments

Male C57/BL6 mice, aged 6-weeks, weighing 16-20 g, were purchased from the Experimental Animal Center of Shandong University. Animal experiments were carried out according to the 'Principles of laboratory animal care' established by the National Institutes of Health, and approved by the 'Animal Care and Use Committee' of the Shandong University (Number: MECSUDMS 2011055). Mice were maintained under diurnal lightning conditions at 25°C with free access to tap water and food. 20 mice were fed with a high-fat diet containing 20% fat and 10 mice were fed with normal diet. Animal body weights were monitored once a week until the difference in body weight between high-fat diet and normal diet mice exceeded 30%. These mice with bigger body weight were regarded as obese mice. The obese mice were randomly divided into two groups: the "control group" and "GH group". GH group mice were injected intraperitoneally (i.p.) with 50 µg of GH per kg of body weight (BW) in 0.2 ml saline once a week for 12 weeks. The control mice were injected with same volume of saline. Prior to injection, all mice were fasted overnight. Finally, after GH treatment, mice were sacrificed by decapitation under anesthesia with 10% chloral hydrate. Epididymal adipose tissues were collected for quantitative real-time PCR analysis.

cDNA microarray analysis

Gene expression profiling was conducted using a microarray chip containing the whole genome array (Boao capital, Beijing, China) for mice (28793 oligoclones). GH treated or non-GH treated 3T3-F442A adipocytes were solubilized in Trizol (Invitrogen, USA) and total RNA was prepared according to the manufacturer's suggested protocol. RNA samples were reverse transcribed and labeled for microarray analysis using standard techniques. Briefly, total RNA was converted to fluorescently labeled with Cy3 or Cy5 and Superscript II reverse transcriptase in the presence of 2-aminoallyl-dUTP as described previously. Imaging and image analysis were performed in a LuxScan 10KA scanner (CapitalBio, Beijing, China). Differentially expressed genes were defined as outliers if the calibrated red to green ratio was greater than 2.0 for all genes that had a minimal intensity of 1500 and a maximal intensity of 6000 in either channel.

Quantitative Real-time PCR (qPCR)

Total RNA was extracted from treated adipocytes as indicated using Trizol reagent (Takara Bio Inc., Dalian, China) and was reversely transcribed into cDNA using PrimeScript RT reagent Kit With gDNA Eraser (Takara Bio Inc.). The qPCR was performed on a Roche LightCycle 480 (Roche, Penzberg, Germany) with an initial enzyme activation step of 3 min at 95 °C, followed by 40 cycles of two-step PCR (95 °C for 45 sec; 60 °C, 60 sec). Primers can be found in Table 1 (Genomics Institute of HuaDa, Beijing, China).

PCR data results were expressed as Ct values, with Ct defined as the threshold

Table 1. Sequence information for the primers used for PCR or qPCR

Genes	Primer sequences for qPCR	Size (bp)
ATGL	5'-TCCGTGGCTGTCTACTAAAGA-3'	118
	5'-TGGGATATGATGACGTTCTCTCC-3'	
Adiponectin	5'-TGTTCTCTTAATCTGCCCA-3'	104
	5'-CCAACCTGCACAAGTTCCTT-3'	
Adipor2	5'-GAGTGTTCGTGGGCTTAGG-3'	143
	5'-GCAGCTCCGGTGATATAGAGG-3'	
C/EBPα	5'-CAAGAACAGCAACGAGTACCG-3'	124
	5'-GTCAGTGGTCAACTCCAGCAC-3'	
FABP	5'-AAGGTGAAGAGCATCATAACCCT-3'	133
	5'-TCACGCCCTTTCATAACACATTCC-3'	
FAS	5'-GGAGGTGGTGATAGCCGGTAT-3'	141
	5'-TGGGTAATCCATAGAGCCAG-3'	
HSL	5'-CCAGCCTGAGGGCTTACTG-3'	106
	5'-CTCCATTGACTGTGACATCTCG-3'	
Leptin	5'-GAGACCCCTGTGTCGGTTC-3'	136
	5'-CTGCGTGTGTGAAATGTCATTG-3'	
PPARγ	5'-GACCACTCGCATTCTTT-3'	360
	5'-GGCATTGTGAGACATCCC-3'	
SOCS2	5'-TCTTTTGGGGTACCCTGTGAC-3'	303
	5'-GAAAGTTCCTTCTGGCGCCTCT-3'	
UCP1	5'-AGGCTTCCAGTACCATTAGGT-3'	133
	5'-CTGAGTGAGGCAAAGCTGATT-3'	
β-Actin	5'-GTGACGTTGACATCCGTAAAGA-3'	245
	5'-GCCGGAATCATCGTACTCC-3'	

cycle of PCR at which amplified PCR product was first detected. To minimize intra- and inter-assay variability generated from differences in PCR efficiency, the quantity of the target sequence was normalized with β -Actin mRNA. The threshold value of the target sequence is directly proportional to the absolute concentration when compared with the Ct value for reference genes. The relative mRNA expression level of the target copies was expressed as fold change compared with control as determined by the $2^{-\Delta\Delta Ct}$ method, a relative quantification algorithm. The factor (X) by which the amount of the changed gene can be calculated with the following formula: $X = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\beta\text{-Actin}})_{\text{sample}} - (Ct_{\text{target gene}} - Ct_{\beta\text{-Actin}})_{\text{control}}$

Reverse transcription PCR

PCR was conducted using the DreamTaq Green PCR Master Mix according to the manufacturer's instructions (K1082, ThermoFisher, Bremen, Germany). PCR products were loaded onto an agarose gel and separated by electrophoresis. The relative semi-quantitative optical density ratio of images was calculated using NIH Image J software, with β -Actin as an internal control.

Protein extraction and Western blotting analysis

After treatment, adipocytes were harvested and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Equal amounts of protein were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and the bands were transferred to polyvinylidene fluoride membrane (Millipore, USA). The membrane was blocked and incubated with the relevant primary antibodies and then incubated with peroxidase-conjugated secondary antibodies (ZhongShanJinQiao, China). Chemiluminescence signals were detected and analyzed with BIO-RAD ChemiDoc MP Imaging System and Image Lab Software. Relative protein levels in each sample were normalized to α -Tubulin to standardize the loading variations.

Enzyme-linked immunosorbent assay (ELISA) for adiponectin

During the process of F442A adipogenesis either with GH intervention or non-GH intervention, the culture mediums were collected and centrifuged (12,000 rpm) for 10 min to remove debris. The supernatants were collected for adiponectin measurements using enzyme-linked immunosorbent assay (Elisa) Kits (cusabio, Wuhan, China) according to the manufacturer's instructions. In brief, standards and samples were pipetted into a 96-well microplate which has been pre-coated with adiponectin antibody. After removing any unbound substances, a biotin-conjugated antibody specific for adiponectin was added to the wells. After washing, avidin conjugated horseradish peroxidase (HRP) was added to the wells. Following a wash to remove an unbound avidin-enzyme reagent, tetramethylbenzidine (TMB) substrate was incubated protect from light in each well and was stopped with H_2SO_4 stop solution. The optical density was measured with a microplate reader (iMark, Bio-Rad, USA) at 450 nm. Relative adiponectin protein levels were normalized to standards.

Statistical analysis

All data are presented as mean $\pm$ SEM from at least three independent experiments. Relative expression of genes, which was described as the fixed factor in our *in vivo* and *in vitro* experiments, is presented as GH-group/Control group. And the each differentiation time point is defined as the random factor. For comparison of quantitative data of two groups, Student's t-test was used, and for multiple comparisons above control due to GH at each time point, one-way ANOVA was used (SPSS17.0 software). $P < 0.05$ was considered statistically significant.

Results

GH modulates the expression of genes related to adipocyte maturation and lipid Synthesis during adipogenesis

During the process of adipogenesis in 3T3-F442A pre-adipocytes, we examined the expression of two adipogenesis regulators (*C/EBP α* and *PPAR γ*) (Fig. 1A and 1B) and two lipid synthesis related genes (*FAS*, *FABP*) (Fig. 1C and 1D), and found that the mRNA expression of *C/EBP α* , *PPAR γ* , *FAS* and *FABP* reached their peaks on day 6 in pre-adipocytes without GH treatment (Fig. 1A-1D). While the mRNA levels of *FAS* and *FABP* were reduced in 3T3-F442A pre-adipocytes treated with GH in different time points (from day 1 to day 12)

Fig. 1. The effect of GH on the expression of adipocyte maturation or lipid accumulation related genes during adipogenesis of 3T3-F442A pre-adipocyte. The mRNA levels of several genes were quantitatively analyzed at different stages during 3T3-F442A pre-adipocytes adipogenesis. (A) *C/EBPα*; (B) *PPARγ*; (C) *FAS*; (D) *FABP*. Results are shown as mean ± SEM of three independent experiments. **P* < 0.05, ***P* < 0.01, GH-treated cells vs. non-GH-treated control cells (NC) at the same time point during the process of adipogenesis.

Fig. 2. The effect of GH on the expression of lipid-lysis or lipid-consuming proteins during adipogenesis of 3T3-F442A pre-adipocyte. The mRNA levels of several molecules were quantitatively analyzed at different stages during 3T3-L1 pre-adipocytes differentiation. (A) *HSL*; (B) *ATGL*; (C) *UCP1*. Results are shown as mean ± SEM of three independent experiments. **P* < 0.05, ***P* < 0.01, GH-treated cells vs. non-GH-treated control cells (NC) at the same time point during the process of adipogenesis.

pocytes differentiation. (A) *HSL*; (B) *ATGL*; (C) *UCP1*. Results are shown as mean ± SEM of three independent experiments. **P* < 0.05, ***P* < 0.01, GH-treated cells vs. non-GH-treated control cells (NC) at the same time point during the process of adipogenesis.

(Fig. 1C and 1D) and the mRNA expression of *C/EBPα* and *PPARγ* were dramatically inhibited 6 days after differentiation (Fig. 1A and 1B).

GH modulates the expression of genes related to lipolysis during adipogenesis

During adipogenesis of 3T3-F442A pre-adipocytes, GH markedly enhanced mRNA expressions of *HSL* and *ATGL* at later time points (Fig. 2A and 2B), and mRNA level of *UCP1* were significantly increased in every different time points (Fig. 2C).

Fig. 3. Western blotting or ELISA analyses the GH impact on protein expression of adipocyte differentiation related genes during the adipogenesis of 3T3-F442A pre-adipocytes. (A) C/EBP α , PPAR γ , FAS and α -Tubulin were western blotted with specific antibodies. (B) Secretion of adiponectin was analyzed with ELISA assay. Results are shown as mean $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, GH-treated cells vs. non-GH-treated control cells (NC) at the same time point during the process of adipogenesis.

GH has the effect on protein expression of C/EBP α , PPAR γ , FAS and adiponectin

As shown in Fig. 3A, GH strongly inhibited the protein expression of C/EBP α , PPAR γ and FAS from day 6 till day 12 during adipogenesis of F442A pre-adipocytes. To determine the role of GH in regulation of adiponectin secretion during the maturation of lipids, the supernatant of cell culture medium were studied. As shown in Fig. 3B, GH significantly increased secretion of adiponectin from day 4. The coefficient variation (CV) of intra-assay was less than 6.5%, and the CV of inter-assay was less than 8%. The data presented in Fig. 3B were inter-assay values analyzed from the means of three parallel intra assays.

GH regulates the expression of genes associated with lipid metabolism in mature 3T3-F442A adipocytes

cDNA microarray analysis showed the gene expression profile (Fig. 4A). 99 genes were showed differential expression (Table 2), 23 genes were showed significantly altered with fold-change values ≥ 2 or ≤ 0.5 in GH-treated mature 3T3-F442A adipocytes compared with control cells. Of the 23 genes, 14 were up-regulated and 9 down-regulated (Table 3). The data of this array have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE86830.

The Gene Ontology (GO) categories showed that 18 genes are associated with lipid metabolism (Table 4). GH inhibited the expression of several genes in fatty acid biosynthesis and elongation pathway at 24 h (Fig. 5).

These results were further confirmed by reverse transcript-PCR (Fig. 4B) and real time RT-PCR for genes, *Adipor2* and *SOCS2* (Fig. 4C and 4D). After the differentiated 3T3-F442A adipocytes were treated with GH (125 ng/mL) for 24 h, the mRNA expression of *Adipor2* was increased by 3-5 folds and the mRNA level of *SOCS2* was increased by 28-folds compared to non-GH treatment control cells.

GH modulates the expression of genes related to lipolysis in differentiated 3T3-F442A adipocytes

Compared with control cells (treated with PBS), GH treatment for 24 h significantly increased the mRNA levels of *adiponectin* and *UCP1*, (Fig. 6C and 6E), while the expressions of *HSL* and *leptin* were not influenced by GH treatment (Fig. 6A and 6D).

Fig. 4. Determination of GH-induced mRNA expression of *SOCS2* and *Adipor2* in 3T3-F442A adipocytes with cDNA microarray, reverse transcription PCR or real-time quantitative PCR (qPCR). (A) Microarray analysis exhibited the expression profile of genes modulated by GH. (B) Reverse transcription PCR showed that GH-induced mRNA expression of *Adipor2* and *SOCS2*. (C,D) GH-induced the expression of *Adipor2* and *SOCS2* were detected and confirmed with qPCR. Results are shown as mean $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, GH-treated cells vs. non-GH-treated control cells (NC).

GH modulates the expression of genes related to lipolysis in primary adipocytes from obese C57/BL6 mice

12 weeks of high-fat diet induced markedly increased in body weight of C57/BL6 mice. As shown in Fig. 7A, compared with control obese mice, GH significantly decreased the mRNA expression of *PPAR γ* , *FAS* and *FABP* in primary adipocytes. While the mRNA level of *HSL*, *ATGL*, *UCP1* and *Adipor2* were increased strongly in GH-treated mice (Fig. 7B).

Discussion

In the present study, we investigated the effects of GH on expression of genes related to lipid accumulation or degradation in 3T3-F442A pre-adipocytes during differentiation, as well as the effect of GH on expression of genes related to adipocyte maturation in 3T3-F442A adipocytes and in obese C57/BL6 mice.

The process of pre-adipocyte differentiation into adipocyte is called adipogenesis. The major features of differentiated adipocytes include morphological change, growth arrest, highly expression of lipogenic genes and production of hormones like leptin. And some adipokines such as leptin and TNF- α may limit the degree of maturation of preadipocytes [12]. *C/EBP α* and *PPAR γ* are two important markers or lipogenic genes for pre-adipocyte transformation into adipocyte, and for adipogenic differentiation from multipotent mesenchymal stem cells to mature adipocytes [15]. After this critical transformation, the adipocyte will accumulate more triglyceride, which is called maturation. Therefore, adipogenesis includes two stages, one is initiation and transformation, the other is maturation.

It was reported that GH induced *C/EBP β* expression may be involved the initiation of the adipocyte differentiation program [16]. Nixon reported that GH presented in serum promoted the conversion 3T3 cells into adipose cells [7]. Kawai reported that GH induced STAT5 pathway stimulated adipogenesis of 3T3-L1 cells in cooperation with *C/EBP β* and *PPAR γ* [17]. But GH stimulated lipolysis has also been documented in animal study [14]. Mc Waters reported transgenic mice with abolished STAT5B activation resulted in obesity [18],

Table 2. 99 differentially expressed genes of Microarray analysis in 3T3-F442A adipocytes treated with GH for 24 H

	Gene Symbol	Ratio	Name	Genebank-accession id
1	<i>Adipor2</i>	5.467	adiponectin receptor 2	AY724514
2	<i>Socs2</i>	4.0799	suppressor of cytokine signaling 2	AF075382
3	<i>Igfals</i>	3.5557	insulin-like growth factor-binding protein complex acid labile chain precursor	BF556234
4	<i>Fxyd5</i>	2.7465	FXD domain-containing ion transport regulator 5 precursor	BC060571
5	-	2.6724	GAAGGGGACCTTGGTCTGTGCCAACCCAGTGATC	
6	<i>Raly</i>	2.4987	TGAGAGTTTCAAGATGCATTAAGATTGGAGCAA	XM_573163
7	-	2.3549	hnRNP-associated with lethal yellow (predicted)	XM_575273
8	<i>Pdlim1</i>	2.3445	TGACACTTCTTGACCAGAGATCCTCTGGTCATAG	
9	<i>Ppp2r5a</i>	2.2872	AACTTGGCCAAAAGCAGGTGATCCAGGTATGTGA	XM_573163
10	<i>Capn6</i>	2.2389	PDZ and LIM domain protein 1	BC072465
11	<i>Clec3b</i>	2.216	similar to protein phosphatase 2	XM_232413
12	<i>Dpt</i>	2.2075	calpain-6	AF067793
13	<i>Obfc2a</i>	2.1981	similar to tetranectin	XM_236746
14	<i>Ap2b1</i>	2.0603	similar to early quiescence protein-1	XM_213925
15	<i>Tmed9</i>	1.9552	Rn30014131	BC089852
16	<i>Ghr</i>	1.94805	adapter-related protein complex 2 beta 1 subunit	M77246
17	<i>Prps1</i>	1.8102	similar to gp25L2 protein	BC088422
18	<i>Cald1</i>	1.8041	growth hormone receptor precursor	J04811
19	<i>St3gal4</i>	1.7849	ribose-phosphate pyrophosphokinase I	BC088149
20	<i>Pten</i>	1.7688	non-muscle caldesmon	NM_013146
21	<i>Shisa4</i>	1.7097	CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase (EC 2.4.99.-)	NM_203337
22	<i>Gadd45g</i>	1.664	phosphatase and tensin	AF017185
23	<i>Acadslb</i>	1.6603	similar to RIKEN cDNA 9330132005	XM_341125
24	-	1.6561	Growth arrest and DNA-damage-inducible protein GADD45 gamma	XM_237999
25	<i>RGD1563150</i>	1.6366	Acyl-CoA dehydrogenase	U64451
26	<i>Ppa1</i>	1.6277	ATGTGAAGAAGTAAATTTGGTCATTCATCTGTGG	
27	<i>Aacs</i>	1.627	GTGTGCGCGTGAACCATCCAGTAGGACACAAACCT	XM_234847
28	<i>Rasl11a</i>	1.6191	similar to B-cell translocation gene 1	XM_233323
29	<i>Lrrc17</i>	1.5988	Pp protein (Fragment)	BC099794
30	<i>Amy1</i>	1.5972	acetoacetyl-CoA synthetase	BC061803
31	<i>Taf15</i>	1.5952	Ras-like family 11 member A	CV111965
32	<i>Iji35</i>	1.5599	leucine rich repeat containing 17	BC079098
33	<i>Rap2b</i>	1.5591	amylase 1, salivary	AB057450
34	<i>Rraga</i>	1.5453	similar to TAF15 RNA polymerase II	XP_237792
35	-	1.5306	interferon-induced protein 35	BC088222
36	<i>Dhx9</i>	1.5302	Ras-related protein Rap-2b	DV216028
37	<i>St3gal1</i>	1.5226	Ras-related GTP-binding protein ragA	BC061850
38	<i>Hagh</i>	1.5197	CCTACATTACTCTGATGAAGATGTCACCAAATACAATGACCTCATCCCGGCA	
39	<i>Fam38a</i>	1.518	GAGAGCAGCAGCCTGACA	XM_340930
40	<i>Gpc4</i>	1.5174	similar to RNA helicase A	XM_239780
41	<i>Syngri1</i>	1.5082	sialyltransferase 4A	AJ748840
42	-	1.5045	hydroxyacylglutathione hydrolase	BC097301
43	<i>Prkrip1</i>	1.5028	similar to Protein FAM38A	XM_341708
44	-	0.6649	Rn30002180	BC081962
45	<i>Kitl</i>	0.6646	synaptogyrin-1 (p29)	U39549
46	-	0.6636	GATGTCTCGGGCCAAGCATCATGCCATCTCTGCTA	
47	<i>Stat3</i>	0.6635	AACTGAACAAGCCCTTCTCTGTTGATACCAAGCCT	NM_172008
48	-	0.6618	similar to PRKR interacting protein 1	XM_573387
49	-	0.6614	CTACCTCTGAAAGATTCTTACAAAAGCGGTGA	
50	<i>roCNC2</i>	0.6576	GTCCCTGTGTGGTATGTTGGGGCAGTGGTGCTG	Rn30022029
51	-	0.655	kit ligand precursor	NM_021843
52	-	0.6524	CAGCTGCTTAAATCTGCTGGATCGCTGTGATCAGC	
53	<i>Porcn</i>	0.6507	CCCTGGCATTGTTAAAAGATATCAGAAGTGTCTTG	XM_243815
54	<i>RGD1309676</i>	0.6494	signal transducer and activator of transcription 3	NM_012747
55	<i>Cdc27</i>	0.6464	CTGTAGGTGCAGTGTCTGCAGGGT	
56	<i>Angptl4</i>	0.6449	GGATCATTACAGAATCAAAAGAGA	XM_213624
57	<i>Gnao1</i>	0.6441	CCCTGGAATGACTATGTGACACAGAGCCCCACAA	
58	<i>Csgalnact1</i>	0.643	TGTTAAATCTGTAGATCCCAATGTTGGCGGACAG	XM_230449
59	<i>Arsj</i>	0.6427	cyclic-nucleotide-gated olfactory channel CNC2 subunit.	U12623
			AGCTGCACCCGTCACATGATGCGTCTGGACATCC	
			GTTCCTGCTACAAGATGCTGCAATTGAAGAAGTA	XM_234416
			CCAGTTGACCTCGTCTCCACGTGCATCGTGAGAC	
			CTTGATAGTTTCCGAACATTTCCCATTTGATAGCT	Rn30026823
			similar to porcupine-D	XM_228781
			Rn30010267	BC079275
			cell division cycle 27 homolog	BC097475
			angiopoietin-related protein 4 precursor	NM_199115
			guanine nucleotide-binding protein G(o)	M17526
			similar to chondroitin beta1,4 N-acetylgalactosaminyltransferase	XM_224757
			arylsulfatase J	XM_227873

continued

while STAT5B activation needs GH and GHR association. It seems that the role of growth hormone in adipocyte differentiation and lipolysis is still controversial.

	Gene Symbol	Ratio	Name	Genebank-accession id
60	<i>Lrp1</i>	0.6416	lrp1_predicted protein	XM_243524
61	<i>Serping1</i>	0.6404	serine (or cysteine) proteinase inhibitor	BC061860
62	<i>Phf17</i>	0.6404	similar to mKIAA1807 protein	XM_227074
63	<i>Lxn</i>	0.6378	latexin	BC081763
64	<i>Slpi</i>	0.6334	secretory leukocyte peptidase inhibitor	CB567769
65	<i>Pms1</i>	0.6332	Rn30003624	BC061722
66	<i>Spp1</i>	0.633	osteopontin precursor (Bone sialoprotein-1) TCGCTCAAGGGATAGACGAACCAATCGTTCCAGTC GCAGTAGAAGTCGAGATAGGCGTAAATGAGGAT AGGACCAATTTATGACCAAACTTATGCTGGTGGAC GGCTGGGTCTGTTTGTCTTCTCCCAAGAAATGGTC TTTCCAGCCTGAAGCCAGCCCATCTCACCTGACAC TGAACAGCGGCTTGGTAGAAAGTGAAGACCTGTGA GTGCCACAGATGGATCCTGCTCCTG	BC078874
67	-	0.6309	CGCTGGCTCCTGCAAATGCAAAACA stromelysin-1 precursor AGAGGGTATTGAGAGATTAATAGGACACGGAGCAG AGGACTCAAGAGCTGACCAGTTTGCCAACAAGTGG	BC091301
68	-	0.6309	similar to yippee-like 3 Rn30004203	XM_214778
69	-	0.6285	Rn30025950 TCCCACCACTGAGCTCCACCCTCCC CGAACTGAACCTAGTCTTTGAACCA	XM_214338
70	-	0.6258	a disintegrin-like and metalloprotease (reprolysin type) endothelin-1 precursor (ET-1) CCTGTATAGTCTCGGCATGTTCCCCACACAGAA CCCACTGTATTTACCTGTCTACTTGTCACCTTTCA	CB313235
71	<i>Mmp10</i>	0.625	keratinocyte growth factor precursor (KGF) embigin precursor protein LRP16 (Fragment) caspase 11 GTGAGTTTCTCATCTAAGCCTAGCGTTTGAACCT GCCAGGCCCAACCTAAATGCAGATTCAAACCCA	XM65253
72	-	0.6224	similar to UDP-N-acetyl-alpha-D-galactosamine TGACCTGCTGGACTGCCTGGTACAGTATCCTACC TGGAAGTGTACAAGGAAGAGTTCGAGATCTGCTC	AY325259
73	<i>RGD1564579</i>	0.6203	similar to hypothetical protein CCTGAAGAGCTTCAAAAACAACCTGGTGGACATCA TTCAGCAGAAACAAGGAGAGGTGGAAGAGTTGGCT	XM_215057
74	<i>RGD1308470</i>	0.6187	Stromelysin-1 precursor FAM3C-like protein ACCCACACACTTCAGTCAGCAGAACTCCTCAAAAG TTTCTAGCCAGCCAAAGGCCAAGAAGATCTCCT	BC087681
75	<i>Abhd12</i>	0.6152	lysosomal acid lipase GGGAAACAGCAGAGGAAGTACCTGGGCAAAGAAAC ATGTGACCTTAAGCCAGATTCTGAAGAATTCTGG	XM_230778
76	-	0.6142	neutrophil gelatinase-associated lipocalin gremlin-1 precursor Rn30015203	Rn30012591
77	<i>Adamts5</i>	0.5943	heme oxygenase 1 nidogen (Entactin) haptoglobin precursor	AY382879
78	<i>Edn1</i>	0.5935		M64711
79	-	0.5918		CB713932
80	<i>Fgf7</i>	0.5915		BG662860
81	<i>Emb</i>	0.5812		BC061846
82	<i>Macrod1</i>	0.5689		AF404762
83	<i>Casp4</i>	0.5686		BC089095
84	-	0.5593		NM_022671
85	<i>Galnt2</i>	0.5588		XM_238057
86	-	0.5464		XM_218828
87	<i>Zcchc11</i>	0.537		XM_233345
88	-	0.5354		AF327838
89	<i>Mmp3</i>	0.53283333		X02601
90	<i>Fam3c</i>	0.5306		AY228475
91	-	0.4929		XM_216295
92	<i>Lipa</i>	0.4892		NM_012732
93	-	0.4861		XM_220070
94	<i>Lcn2</i>	0.4443		BC089053
95	<i>Grem1</i>	0.4381		Y10019
96	<i>Wwtr1</i>	0.3517		BC097968
97	<i>Hmox1</i>	0.3478		NM_012580
98	<i>Nid1</i>	0.3442		XM_213954
99	<i>Hp</i>	0.2672		AF476963

It has been reported that the mRNA expression of 22 genes associated with lipid metabolism was modulated in 3T3-F442A adipocytes in response to GH for 48 h by exploiting microarray analysis [19]. Our microarray data showed that the mRNA expression of 18 genes associated with lipid metabolism changed in mature 3T3-F442A adipocytes upon GH treatment for 24 h. Among these 18 genes, *Adipor2* and *Fads1* were up-regulated, and genes such as *Scd2*, *Ltc4s*, *Acox1* and *Scd1* were down-regulated by GH. These genes are overlapped with the published data and the trend of these genes' expression in response to GH is in concert with the published data [19]. It is also concluded by Jessica Schwartz's group that prolonged GH treatment inhibits genes associated with lipid and cholesterol biosynthesis [19]; in their report, some genes involved in fatty acid synthesis or cholesterol synthesis pathway were reduced in mRNA expression in 3T3-F442A adipocytes upon GH treatment for 48 h [19]. As well as in the published article, our microarray data showed GH down-regulates *Scd1* and *Scd2*, which are closely related to unsaturated fatty acid (oleic acid) biosynthesis. Our microarray data also exhibited that GH increased the expression of acyl-CoA dehydrogenase (*Acadsb*) which involved in the pathway for fatty acid β -oxidation; though the increased fold is not so big, potentially GH has the impact on fatty acid clearance. Besides, our microarray data also showed that GH down-regulated *Fabp4*, which is essential

Table 3. Microarray analysis of modulated genes in 3T3-F442A adipocytes treated with GH for 24 h (fold-change values ≥ 2 or ≤ 0.5)

Name	Ratio	Oligo id	Genebank-accession id
<i>Adipor2</i>	5.467	Rn30007263	AY724514
<i>SOCS2</i>	4.0799	R002975_01	AF075382
<i>Igfals</i>	3.5557	Rn30013818	BF556234
<i>Fxyd5</i>	2.7465	Rn30019436	BC060571
-	2.6724	GAAGGGGACCTTGGTCTGTGCCAACCCAGTGATCTGAGAGTTCAG AAGTGCATTAAAGATTGGAGCAA	
<i>Raly</i>	2.4987	Rn30016005	XM_575273
-	2.3549	TGACACTTCTCTGACCAGAGATCCTCTGGTCATAGAAGTGGCCAA AAGCAGGTGATCCCAAGGTATGTGA	
<i>Pdlim1</i>	2.3445	R002183_01	BC072465
<i>Ppp2r5a</i>	2.2872	Rn30000051	XM_232413
<i>Capn6</i>	2.2389	R000399_01	AF067793
<i>Clec3b</i>	2.216	Rn30004060	XM_236746
<i>Dpt</i>	2.2075	Rn30002616	XM_213925
<i>Obfc2a</i>	2.1981	Rn30014131	BC089852
<i>Ap2b1</i>	2.0603	R004098_01	M77246
-	0.4928	ACCCACACACTTCAGTCAGCAGAACTCCTCAAAGTTTCTAGCCAGC CAAAGGCCAAAGAAGATCTCCT	
<i>Lipa</i>	0.4892	GGGAAACAGCAGAGGAAGTACCTGGGCAAGAAACATGTGACCCTA AGCCAGATTCTGAAGAATTCTGG	NM_012732
-	0.4861	Rn30017550	
<i>Lcn2</i>	0.4443	R002276_01	BC089053
<i>Grem1</i>	0.4381	Rn30024343	Y10019
<i>Wwtr1</i>	0.3517	Rn30015203	BC097968
<i>Hmox1</i>	0.3478	R000442_01	NM_012580
<i>Nid1</i>	0.3442	Rn30002219	XM_213954
<i>Hp</i>	0.2672	R002022_01	AF476963

Table 4. Genes associated with lipid metabolism change in mRNA expression in 3T3-F442A adipocytes upon GH treatment for 24 h

Gene Symbol	Ratio	Name	Genebank-accession id
<i>Adipor2</i>	5.467	adiponectin receptor 2	AY724514
<i>SOCS2</i>	4.0799	suppressor of cytokine signaling 2	AF075382
<i>Igfals</i>	3.5557	insulin-like growth factor-binding protein	BF556234
<i>Acadsh</i>	1.6603	acyl-Coenzyme A dehydrogenase	U64451
<i>Aacs</i>	1.627	acetoacetyl-CoA synthetase	BC061803
<i>Fads3</i>	0.8601	fatty acid desaturase 3	AJ494720
<i>Acaa2</i>	0.8483	acetyl-CoA acyltransferase 2	X05341
<i>Gcdh</i>	0.8466	glutaryl-CoA dehydrogenase	XM_344744
<i>Hadh</i>	0.8447	3-hydroxyacyl-CoA dehydrogenase	AF069770
<i>Ppap2a</i>	0.8406	lipid phosphate phosphohydrolase 1	CF110609
<i>Dhhdh</i>	0.8172	enoyl-CoA hydratase	BC089777
<i>Scd2</i>	0.8159	stearoyl-CoA desaturase 2	AB032243
<i>Ltc4s</i>	0.7619	leukotriene C4 synthase	AB048790
<i>Acaca</i>	0.7579	acetyl-CoA carboxylase 1	J03808
<i>Fabp3</i>	0.7434	fatty acid binding protein 3	CB698942
<i>Acox1</i>	0.7388	acyl-CoA oxidase 1, palmitoyl	J02752
<i>Fabp4</i>	0.7005	fatty acid binding protein 4	BC074002
<i>Scd1</i>	0.6699	stearoyl-CoA desaturase 1	J02585

Fig. 5. Schematic presentation of the genes down-regulated by GH on lipid acid biosynthesis pathway at 24 h analyzed from microarray data. Each number stands for a specific gene which is listed on the right.

for fatty acid transporting into adipocytes; therefore, GH might reduce the triglycerol synthesis in adipocytes. In a word, our data confirmed the conclusion about the impact of GH on lipid metabolism in the published research article [19], though we chose a different GH

Fig. 6. Quantitative analyses the expression of lipolysis related genes at mRNA level in mature 3T3-F442A adipocytes treated with GH. (A) *HSL*; (B) *ATGL*; (C) *adiponectin*; (D) *leptin*; (E) *UCP1*. Results are shown as mean $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, GH-treated cells vs. non-GH-treated control cells (NC).

Fig. 7. Effect of GH on the expression of lipid or lipolysis related genes in vivo. Epididymal adipose tissues were separated from the high-fat diet induced-obese C57/BL6 mice. GH intervention mice were injected with GH (50 μ g/kg. BW), and control mice were injected with saline (n = 6). (A) Quantitative analyses the expression of lipid accumulation related genes at mRNA level; (B) Lipolysis related genes at mRNA level. Results are shown as mean $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, GH group (GH) vs. control (NC).

prolonged treatment time point (24 h). Our microarray data also showed GH is able to reduce the expression of *Angptl4* (angiotensin-like protein 4, ratio 0.6449), *Angpt1* (angiotensin 1, ratio 0.5285) or *Lipa* (lysosomal acid lipase, ratio 0.4892) in 3T3-F442A adipocytes, the

biological significance of which needs to be further studied.

It has been reported that SOCS2 overexpression significantly inhibited the expression of fatty acid synthase (FAS), the mRNA expression of adipose triglyceride lipase (ATGL), and the protein expression in swine primary adipocytes [20]. Besides, GH activated STAT5B, which in turn induces SOCS2 expression, finally SOCS2 inhibits insulin activation which is critical for adipogenesis and lipid accumulation. Our data clearly showed that GH in differentiated adipocytes increased the expression of *SOCS2*, indicating that GH potentially inhibits fatty acid synthesis and triglyceride synthesis in adipocytes.

Adiponectin is the most abundant peptide derived by adipose, play significant role in the etiology of obesity-related chronic diseases, such as insulin resistance and type 2 diabetes [21]. Adiponectin has been reported to reduce the expression of PPAR γ , FABP4, FAS, thereby reduce lipid content [22]. When adiponectin binds to its receptors (Adipor1/2), it activates adenosine 5'-monophosphate-activated protein kinase (AMPK), promoting glucose uptake by skeletal muscle via intracellular translocation of the glucose transporter type 4 (GLUT4). Animal studies showed that *Adipor1* transgene mice enabled mice to resist high fat/high sucrose diet-induced obesity while decreasing lipid accumulation [23]. We found that in mature F442A adipocytes, GH treatment increased the abundance or availability of adiponectin receptors, and increased adiponectin protein expression. Our animal study also confirmed that GH intervention increased the mRNA level of *Adipor2* in epididymal adipose tissue from obese mice.

C/EBP α is the initial transcriptional factor induced by adipogenic hormone, and is thought to be required for PPAR γ expression [24]. C/EBP α and PPAR γ promoted differentiation by activating adipose-specific gene expression and by maintaining each other expression at high levels. In our adipogenesis system, C/EBP α and PPAR γ protein expression levels reached their peaks at 4 day post initiation of differentiation of pre-adipocytes.

Insulin is an essential hormone for adipogenesis. GH activates PI3K and ERK signaling pathways similar to that of insulin, therefore, GH was suggested to have similar effects on the initiation and transformation of pre-adipocyte differentiation as insulin [7, 8]. Our previous data showed insulin and GH has synergistic effects on GH induced STAT5 activation [9]. In the present study, We found that in mature adipocyte, GH treatment reduced the expression of C/EBP α and PPAR γ , which indicated that GH has potentially inhibitory effect on lipid accumulation of adipocytes or adipocyte maturation. During adipogenesis, GH intervention resulted in reduced mRNA and protein expression of C/EBP α and PPAR γ at day 4 after the initiation of adipogenesis, which indicated that GH has inhibitory effect on adipocyte maturation. We also checked the expression levels of C/EBP β and PPAR α in parallel with C/EBP α and PPAR γ during the process of GH intervention in adipogenesis, but no significance changes were detected (data not shown). Waxman once reported STAT5B activation inhibited PPAR γ transactivation [25], while STAT5B is a downstream signaling molecule of GH receptor. It is possible that GH regulates PPAR γ expression by multiple pathways.

FAS and FABP are two genes directly related to adipocyte lipid synthesis. Our data showed that GH intervention during lipogenesis significantly reduced the mRNA levels of *FAS* and *FABP*, GH intervention markedly reduced the protein expression of FAS at day 4 after the initiation of adipogenesis. In mice epididymal adipose tissue, the mRNA levels of *FAS* and *FABP* were also decreased significantly with GH intervention. These data indicates that GH inhibits adipocyte maturation and lipid synthesis in mature adipocytes.

The biological role of UCP1 is to uncouple ATP synthesis from respiratory chain, leading to thermal heat production or energy storage reduction in adipose tissue in small mammals and human infants [26]. UCP1 is "brown-fat-specific" and converts nutrient energy directly to heat in brown adipose tissue. In this present study, we found that during the maturation process of F442A adipocytes and in primary adipose tissue, GH intervention markedly increased *UCP1* expression, which indicated that GH might endows F442A adipocytes or adipocytes in adipose tissues some features of brown adipocytes.

To determine whether GH is involved in lipolysis, we examined its effects on the expression of HSL and ATGL. HSL is a rate-limiting enzyme for lipolysis, while ATGL plays

essential role in the initial step of triglyceride hydrolysis [27]. During adipogenesis of F442A pre-adipocytes, GH treatment showed no obvious effect on the expression of *HSL* or *ATGL*. Of note, in animal study, we found that GH was able to enhance the expression of *HSL* and *ATGL* in adipose tissues. It is possible that GH may have no direct effect on *ATGL* and *HSL* expression, but could increase *ATGL* and *HSL* expression *in vivo* via an indirect way.

In summary, we found that during the process of adipogenesis, GH decreases the expression of adipogenic genes and increases the expression of *UCP1* and adiponectin, which is likely to reduce lipid storage. In mature adipocytes, GH reduces the expression of important lipid-accumulation related genes such as *C/EBPα*, *PPARγ*, *FAS* and *FABP*, but enhances the expression of lipid reduction genes such as *adipor2*, *SOCS2* and adiponectin. These results indicate that GH negatively modulates adipocyte maturation and lipid accumulation.

Abbreviations

Adipor1/2 (adiponectin receptor 1/2); *ATGL* (adipose triglyceride lipase); *CIS* (cytokine-inducible SH2 containing protein); *C/EBPα/β* (CCAAT/enhancer binding protein α/β); *FABP* (fatty acid binding protein); *FAS* (fatty acid synthase); *GH* (growth hormone); *HSL* (hormone-sensitive lipase); *PPARα/γ* (peroxisome proliferator-activated receptor α/γ); *SOCS* (suppressor of cytokine signaling); *UCP1* (uncoupling protein 1); *CoA* (coenzyme A).

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Disclosure Statement

The authors declare that they have no competing interests.

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