Growth hormone stimulates adipogenesis of 3T3-L1 cells through activation of the Stat5A/5B-PPAR γ pathway

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Abstract

Growth hormone-deficient (GHD) patients show a decreased number of adipocytes, which is normalized by GH replacement, indicating an adipogenic effect of GH. However, the precise mechanisms underlying this effect remain to be clarified. In this study, we investigated the adipogenic effect of GH. GH stimulated MDI (3-isobutyl-1-methylxanthine, dexamethasone, and insulin)-induced adipogenesis of 3T3-L1 cells with early induction of peroxisome proliferator-activated receptors (PPAR) γ 2 expression. This adipogenic effect of GH was suppressed by overexpression of Stat5A mutant (Stat5A-Y694F), a transcriptional suppressor for the GH-Stat5A/5B signaling pathway, with the reduction of PPAR γ 2 expression. Next, we investigated the relationship between Stat5A/5B and CCAAT/enhancer binding protein (C/EBP) β/δ or PPAR γ in 3T3-L1 cells. Stat5A/5B stimulated C/EBP β - and C/EBP δ -induced adipogenesis with enhancement of PPAR γ 2 expression. In addition, Stat5A/5B enhanced the transcriptional activity of C/EBP β/δ in the PPAR γ gene promoter. Furthermore, Stat5A/5B stimulated PPAR γ -induced adipogenesis and enhanced the transcriptional activity of PPAR γ . These results suggest that the GH-Stat5A/5B signaling pathway stimulates adipogenesis in cooperation with C/EBP β/δ and PPAR γ . To completely understand the effect of GH, cDNA microarray analysis was performed to screen genes affected by GH during MDI-induced adipogenesis. Among 4277 genes, 18 and 19 genes were up- and down-regulated respectively. cDNA microarray analysis also indicated the up-regulation of PPAR γ and the modulation of expression of genes coding for growth factors or growth factor receptors, suggesting that GH stimulates adipogenesis in association with the modulation of cell growth. Thus, the GH-Stat5A/5B signaling pathway stimulates adipogenesis through two distinct steps. In addition, cDNA microarray data provide us the further insights underlying the adipogenic effect of GH.

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Introduction

Growth hormone (GH) is not only a major regulator of postnatal somatic growth, but also has an important role in the control of body composition and fat distribution, through the combination of anabolic, lipolytic, and anti-natriuretic actions (Isaksson *et al.* 1982, Nam & Lobie 2000, Tollet-Egnell *et al.* 2004, Rowland *et al.* 2005). Indeed, GH-deficient (GHD) patients exhibit a decreased number of adipocytes and an increased mean volume of adipocytes, and this abnormal adipose tissue composition is normalized by GH replacement (Bonnet *et al.* 1974, Salomon *et al.* 1989, Bengtsson *et al.* 1993). As GH replacement increases the number of adipocytes in GHD patients, this indicates a stimulatory effect of GH on adipogenesis. To date, adipogenic activity of GH has been reported in studies *in vitro* (Morikawa *et al.* 1984, Nixon & Green 1984). GH is strictly required in the conversion of preadipocytes to adipocytes and is thought to play a

role in priming the cells to become responsive to insulin and insulin-like growth factor-I (IGF-I) in 3T3-F442A cells (Guller *et al.* 1989, Corin *et al.* 1990, Wabitsch *et al.* 1995). In other cell lines, GH also stimulates adipogenesis, although the role of GH is not exclusive (Doglio *et al.* 1986, Tominaga *et al.* 2002). However, little is known about the precise mechanisms of the stimulatory effect of GH on adipogenesis.

Adipogenesis is a well-regulated process controlled by the sequential activation of various transcription factors (Rosen & Spiegelman 2000). Adipogenic hormones, including insulin, glucocorticoids, and cAMP-producing factors, and fetal calf serum (FCS) allow growth-arrested preadipocytes to re-enter the cell cycle and undergo approximately two rounds of mitosis called mitotic clonal expansion. Both CCAAT/enhancer-binding protein (C/EBP) β and C/EBP δ are transiently induced during this period. These transcription factors induce the expression of peroxisome proliferator-activated receptor (PPAR) γ . PPAR γ in turn stimulates the expression of

C/EBP α . Subsequently, PPAR γ and C/EBP α positively activate the transcription of each other. Concurrently, adipogenesis enters the terminal differentiation stage, and adipocyte-specific genes including aP2, leptin, and adiponectin begin to be expressed. Although C/EBP β and C/EBP δ have an important role in adipogenesis, mice lacking both C/EBP β and C/EBP δ exhibited normal PPAR γ expression in adipose tissue, indicating that PPAR γ expression is maintained through several pathways (Tanaka *et al.* 1997). In addition, PPAR γ null cells showed no adipogenic potential (Rosen *et al.* 1999). Combined together, PPAR γ rather than C/EBP β/δ has the essential role in adipogenesis.

Signal transducer and activator of transcription Stat5A and 5B, both of which have an approximately 90% identical sequence at amino acid levels, are key transducers of the GH signaling pathway (Darnell 1997). When GH binds to GH receptor (GHR), cytoplasmic tyrosine kinase Janus kinase 2 (JAK2) associates with the cytoplasmic domain of GHR and phosphorylates Stat5A/5B. Phosphorylated Stat5A/5B dimerizes, translocates into the nucleus and binds to the DNA (Piwien-Pilipuk *et al.* 2002). Stat5A/5B-deficient mice showed reduced weight, similar to GH- or GHR-deficient mice, and had approximately one-fifth the epidermal fat pad of wild-type mice, indicating that GH may exert its effect on adipose tissue formation through Stat5A/5B (Teglund *et al.* 1998). Consistent with this, several investigators have reported the contribution of Stat5A to adipogenesis in some cell lines (Yarwood *et al.* 1999, Nanbu-Wakao *et al.* 2002, Floyd & Stephens 2003, Shang & Waters 2003, Stewart *et al.* 2004). However, the precise mechanisms by which Stat5A promotes adipogenesis have not been completely established. Furthermore, the role of Stat5B in adipogenesis remains to be clarified.

In addition to transcription factors and signaling transducers known to be involved in adipogenesis, it is necessary to examine the expression pattern of a number of genes to completely understand the effect of GH on adipogenesis. To achieve this purpose, we performed cDNA microarray analysis to screen the gene profiles during the course of GH-mediated adipogenesis of 3T3-L1 cells.

In this study, we attempted to elucidate the role of Stat5A and 5B in the stimulatory effect of GH on adipogenesis, and demonstrated that GH exerts its effect through Stat5A and 5B, which stimulates PPAR γ expression. Furthermore, we revealed that Stat5A and 5B enhance C/EBP β -, C/EBP δ -, and PPAR γ -induced adipogenesis. Interestingly, Stat5A and 5B enhanced the transcriptional activity of C/EBP β/δ and PPAR γ . Our results provide the evidence that the GH-Stat5A/B signaling pathway stimulates adipogenesis by two distinct steps through stimulating C/EBP β/δ and PPAR γ activities.

Materials and methods

Materials

3T3-L1 cells and C3H10T1/2 cells were obtained from the Human Science Research Resources Bank (Osaka, Japan). Growth hormone, insulin, 3-isobutyl-2-methylxanthine, and cycloheximide were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Dexamethasone and triiodothyronine were purchased from ICN Biomedicals, Inc. (Aurora, OH, USA). Biotin, epidermal growth factor (EGF), fetuin, and anti- β -actin antibody were purchased from Sigma. The anti-PPAR γ antibody, anti-C/EBP β antibody, and anti-C/EBP δ antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and the anti-Stat5 antibody and anti-proliferating cell nuclear antigen (PCNA) antibody were from BD Transduction Laboratory, Inc. (Lexington, KY, USA).

Cell culture

3T3-L1 cells and C3H10T1/2 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FCS in 5% CO $_2$ at 37 °C. For the induction of adipogenesis, 3T3-L1 cells were grown to confluence. Two days after confluence, the medium was changed to differentiation medium containing 1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 μ g/ml insulin (MDI) in serum-free medium consisting of 5 μ g/ml transferrin, 2 nM triiodothyronine, 30 ng/ml EGF, 1 μ M biotin, and 200 μ g/ml fetuin in DMEM (Tominaga *et al.* 2002). After treatment with differentiation medium, the medium was changed to maintenance medium (serum-free medium).

Oil Red O staining

On day 6, cells were stained with Oil Red O as follows. The cells were washed with PBS and fixed with 10% formalin in PBS for 20 min. After fixing, cells were washed twice with PBS and once with 60% isopropyl-alcohol. The cells were stained with 60% Oil Red O solution from a stock of 0.25 g Oil Red O in 50 ml isopropyl-alcohol for 1 h (Sigma). To quantify the incorporation of lipid, the area stained with Oil Red O was measured using an Image Pro Plus analyzer (MediaCybernetics, Inc., Silver Spring, MD, USA).

Constructs and luciferase reporter assay

pcDNA1 Stat5A expression vector, pcDNA1 Stat5B expression vector, and β -casein promoter linked to a luciferase reporter gene (*pZZ1-Luc*) were kindly

provided by Yamashita *et al.* (2003). Stat5A-Y694F was generated by substituting phenylalanine for tyrosine at 694 amino acid and subcloned into a pcDNA1 expression vector (Invitrogen Life Technologies). The sequence of the mutant cDNA was confirmed by DNA sequence analysis. GHR cDNA (pMet-IG-mGHR) was a kind gift from Dr John J Kopchick (Ohio University, Ohio). pCMX-PPAR γ expression vector and PPAR γ -binding element (PPRE)-luciferase construct (PPREx3-TK-Luc) were described previously (Kliwer *et al.* 1994). C/EBP β cDNA and C/EBP δ cDNA were kindly provided by Dr Shizuo Akira (Tanaka *et al.* 1997). PPAR γ promoter fused to a luciferase construct was described previously (Ichida *et al.* 2004). Transient transfection was carried out using FuGENE 6 (Roche Applied Science) following the manufacturer's protocol. The total amount of DNA added to each well was equalized using an empty vector. Luciferase assay was performed in duplicate according to the protocol of the dual-luciferase reporter assay system (Promega). Briefly, 2 days after transfection, cells were lysed and luciferase activity was determined using specific substrates in a luminometer. Transfection efficiency was normalized by co-transfection with TK-*Renilla* luciferase construct (Promega).

Generation of adenovirus

Adenoviruses carrying Stat5A, Stat5B, Stat5A-Y694F, C/EBP β , C/EBP δ and PPAR γ were constructed using ViraPower Adenoviral Expression System (Invitrogen Life Technologies). Briefly, cDNA was inserted into TOPO pENTR vector and was recombined to the adenovirus expression plasmid pAd/CMV/V5-DEST. The pAd/CMV/V5-DEST plasmid with cDNA was digested with the PacI endonuclease and transfected with HEK293A cells. The medium supernatant containing adenovirus was collected and titrated according to the manufacturer's instructions. At 100% confluency, 3T3-L1 cells were infected with adenovirus at multiplicity of infection (MOI) of 500 with 0.5 μ g/ml poly-L-lysine (Sigma; Orlicky & Schaack 2001) in DMEM containing 2% FCS for 6 h. C3H10T1/2 cells were infected with adenovirus at MOI of 200 in DMEM containing 10% FCS.

Western blot analysis

To prepare whole cell lysates, cells were washed twice with ice-cold PBS and solubilized in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 2 μ g/ml pepstatinA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Lysates were centrifuged for 20 min at 4 °C at 12 000 *g* and

supernatants were collected. For cell fractionation, 3T3-L1 cells were washed twice with ice-cold PBS and solubilized in hypotonic lysis buffer (10 mM Hepes (pH 7.8), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Lysates were centrifuged at 12 000 *g* for 20 min and supernatants were used as cytosol extracts. The pellet was then lysed with hypertonic buffer (50 mM Hepes (pH 7.8), 420 mM KCl, 0.1 mM EDTA (pH 8.0), 5 mM MgCl₂, 2% glycerol, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) and supernatants collected after centrifugation (12 000 *g* for 20 min) were used as nuclear extracts. The protein concentration was determined by the Lowry method using the DC reagent (Bio-Rad Laboratories). The same amount of samples was separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The amount of protein was checked by Ponceau S staining. Membranes were blocked in 5% BSA in Tris-buffered saline (TBS). Thereafter, the membranes were immunoblotted with anti-C/EBP β (1:250), anti-C/EBP δ (1:250), anti-PPAR γ (1:250), anti-Stat5 (1:250), anti- β -actin (1:2000), or anti-PCNA (1:5000), and developed with horseradish peroxidase-coupled anti-mouse IgG antibodies, followed by enhancement with ECL detection kits (Amersham Biosciences) or SuperSignal West Dura Extended Duration Substrate Antibodies (Pierce Chemical Co., Rockford, IL, USA). The protein bands were digitally imaged for densitometry using Scion software (Scion Corp., Frederick, MA, USA).

RNA preparations and real-time reverse transcription-PCR (RT-PCR)

Total RNA was prepared with RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, and cDNA was generated using a random primer and reverse transcriptase (Superscript II, Invitrogen Life Technologies) according to the manufacturer's instructions. Quantification of mRNA expression was carried out using a LightCycler amplification and detection system (a LightCycler-FastStart DNA Master SYBR Green I; Roche Diagnostics). *GAPDH* gene was used as an internal standard gene for quantification. PCR product of each gene was inserted into a pGEM T vector (Promega) and used as a standard dilution with a known number of copies. Conditions of amplification were: an initial step (94 °C, 10 min) followed by 35 cycles consisting of a denaturation step (94 °C, 15 s), primer annealing (55 °C for PPAR γ 2, 57 °C for glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*), 5 s) and an extension step (72 °C, 10 s). The products for PPAR γ 2 and *GAPDH* were detected at 85 °C.

Specific primers for PPAR γ 2 (forward primer: 5'-GGTGAACTCTGGGAGATTC-3'; reverse primer: 5'-CAACCATTGGGTCAGCTCTT-3'), GAPDH (forward primer: 5'-TGAACGGGAAGCTCACTGG-3'; reverse primer: 5'-TCCACCACCCTGTTGCTGTA-3').

cDNA microarray analysis

The quality of extracted total RNA was confirmed with an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). An IntelliGene II Mouse CHIP (Takara, Shiga, Japan), on which 4277 mouse genes were spotted, was utilized to perform the cDNA microarray analysis. Cy3- and Cy5-labeled cDNA were prepared with mRNA isolated from control cells and GH-treated cells. Both were mixed and hybridized to the CHIP. The hybridized CHIP was scanned using an Affimetrix 418 Array Scanner (Woburn, MA, USA) and ImaGene software (BioDiscovery, Los Angeles, CA, USA). The experiments were performed thrice independently. To confirm the data, RT-PCR was performed using Taq DNA Polymerase (Promega) and specific primers for interleukin-4 (IL-4) receptor (forward primer: 5'-CTGTGGGCTGTCTGATTTTG-3'; reverse primer: 5'-CTGGGTTTCCTTGTAAGTCAC-3'), vascular endothelial growth factor (VEGF) A (forward primer: 5'-TGTGCAGGCTGCTGTAACGA-3'; reverse primer: 5'-CGCCTTGGCTTGTACATCT-3'), TWIST (forward primer: 5'-ATGGACAGTCTAGAGACTCTG-3'; reverse primer: 5'-GCATTTTACCATGGGTCATC-3'), and β -actin (forward primer: 5'-TGGGAATGGGTCAGAAAGACTC-3'; reverse primer: 5'-AGAGGCATACAGGGA-CAGCACA-3'). β -Actin was used as an internal standard.

Statistical analysis

All data are expressed as the mean $\pm$ S.E.M. Results were analyzed for statistically significant differences using Student's *t*-test or ANOVA followed by Bonferroni multiple comparison *post hoc* test (Stat View software, SAS Institute, Cary, NC, USA). Statistical significance was set at $P < 0.05$.

Results

GH stimulates adipogenesis of 3T3-L1 cells

In order to investigate the effect of GH on adipogenesis, 3T3-L1 cells were treated with 50 ng/ml GH in the presence of 10% FCS as the first experimental condition. GH could not induce adipogenesis, indicating that GH by itself has little effect on the initiation of adipogenesis (Fig. 1A). Next, we treated 3T3-L1 cells with differentiation medium for 2 days, and 50 ng/ml GH were added to both differentiation and maintenance media to

examine the effect of GH. Since GH is intrinsically contained in FCS, serum-free medium was utilized (Smith *et al.* 1988). On day 6, the area of Oil Red O staining did not show any significant difference between cells treated with or without GH (Fig. 1B). Treating cells with differentiation medium for 2 days is the most commonly used protocol for adipogenesis (Rubin *et al.* 1978). In this condition, most cells were strongly induced to differentiate into adipocytes even in serum-free medium (Fig. 1B). We speculated that such a potent induction protocol is likely to mask the effect of GH on adipogenesis. In order to detect the effect of GH, we modified the induction protocol by reducing the incubation time with differentiation medium. When cells were treated with differentiation medium for 3 h followed by incubation with maintenance medium, addition of GH to both differentiation and maintenance media showed the significant initiation of adipogenesis when compared with control (Fig. 1B and C). The effect of GH was in a dose-dependent manner and 50 ng/ml GH showed the maximum effect on adipogenesis (Fig. 1D). Although more than 50 ng/ml GH showed the trend of decrease in GH effect, there was no significant difference.

GH stimulates adipogenesis of 3T3-L1 cells through the early induction of PPAR γ 2 expression, not C/EBP β and C/EBP δ

To examine whether GH has any effects on the expression of C/EBP β , C/EBP δ and PPAR γ , we determined the expression of these transcription factors under the culture condition in which GH shows its stimulatory effect on adipogenesis. Although both C/EBP β and C/EBP δ were transiently induced after 3 h adipogenic induction, GH did not affect the expression of C/EBP β and C/EBP δ (Fig. 2A). PPAR γ has two isoforms which are generated by alternative splicing of mRNA (PPAR γ 1 and PPAR γ 2). Whereas PPAR γ 1 is expressed in several tissues including adipose tissue, PPAR γ 2 is specifically expressed in adipose tissue (Vidal-Puig *et al.* 1997). PPAR γ 1 was expressed in 3T3-L1 preadipocytes, and the expression of PPAR γ 1 increased gradually as adipogenesis proceeded (Fig. 2A). PPAR γ 2 was not expressed in 3T3-L1 preadipocytes, and the expression of PPAR γ 2 increased gradually as adipogenesis proceeded, and was induced earlier in cells treated with GH when compared with cells without GH (Fig. 2A and B). Quantitative RT-PCR analysis also showed a significant up-regulation of PPAR γ 2 mRNA expression in the cells treated with GH (Fig. 2C). To examine whether this enhanced PPAR γ expression is due to increased synthesis or decreased degradation, 3T3-L1 cells were transfected with PPAR γ expression vector and cultured with or without GH for 16 h. After incubation with GH, 10 μ g/ml cycloheximide was added to inhibit

Figure 1 Stimulatory effect of GH on adipogenesis. (A) 3T3-L1 cells were incubated with or without 50 ng/ml GH in DMEM containing 10% FCS for 14 days, and stained with Oil Red O. No adipocytes were observed. (B and C) 3T3-L1 cells were treated with differentiation medium for either 2 days or 3 h followed by incubation with maintenance medium. GH was added to both differentiation and maintenance media. On day 6, the cells were stained with Oil Red O (B) and the stained area was measured in cells treated with 3-h MDI (C). While 2-day treatment did not show any significant difference between cells treated with or without GH, 3-h treatment showed the stimulation of adipogenesis in the cells treated with GH. (D) Dose-dependent manner of GH effect. 3T3-L1 cells were induced to differentiate with 3-h MDI treatment and GH was added at the indicated concentration (0–200 ng/ml). On day 6, the cells were stained with Oil Red O and the stained area was measured. Experiments were repeated independently five times. Statistically significant difference was: * $P < 0.001$ and $^{\dagger}P < 0.05$.

de novo protein synthesis, and whole cell lysates were collected at indicated periods. Western blot analysis of PPAR γ showed no difference in the degradation rate between cells treated with or without GH (Fig. 2D). These findings suggest that GH exerts its effect through the up-regulation of PPAR γ synthesis.

GH facilitates the nuclear translocation of Stat5 during the early stage of adipogenesis

To address whether Stat5A and 5B are implicated in GH-mediated adipogenesis, we first examined the intracellular localization of Stat5 by subcellular fractionation. Consistent with the previous report (Richter *et al.* 2003), localization of Stat5 in the nucleus significantly increased in cells treated with GH at 3, 6, and 12 h (Fig. 3A

and B). Immunofluorescence experiments also showed nuclear localization of Stat5 as early as 1 h after GH stimulation (data not shown). As shown in Fig. 3B, in 3T3-L1 cells treated with GH, nuclear Stat5 expression increased at 3 h, significantly decreased thereafter and increased after 24 h. Similar to our data, Nanbu-Wakao *et al.* (2002) reported the tyrosine phosphorylation of Stat5 during adipogenesis using 3T3-L1 cells treated with standard 2-day MDI in the presence of 10% FCS. In contrast, in 3T3-L1 cells treated without GH, nuclear Stat5 expression increased after 24 h, indicating that Stat5 is activated independent of GH in this condition (Fig. 3B, open columns). Component of maintenance medium or growth factor(s) produced by 3T3-L1 cells during adipogenesis may be involved in Stat5 activation. Indeed, EGF, which is a component of maintenance

Figure 2 Effect of GH on the expression of C/EBP β , C/EBP δ , and PPAR γ . 3T3-L1 cells were induced to differentiate by 3-h incubation with differentiation medium followed by incubation with maintenance medium, and 50 ng/ml GH were added to both differentiation and maintenance media. (A) Nuclear extracts were collected at the indicated periods and then subjected to western blot analysis of PPAR γ , C/EBP β , C/EBP δ and PCNA. Earlier induction of PPAR $\gamma 2$ expression was noted in the cells treated with GH. (B) The protein expression of PPAR $\gamma 2$ was quantified by densitometry from four independent experiments. Densities were normalized against the density of PCNA. (C) Quantitative RT-PCR analysis of mRNA expression of PPAR $\gamma 2$. Total RNA was extracted 3 h after adipogenic induction. Data were normalized by the expression of GAPDH. (D) Degradation of PPAR γ . Whole cell lysates of 3T3-L1 cells transfected with PPAR γ expression vector were subjected to western blot analysis of PPAR γ and β -actin at the indicated periods after the addition of 10 μ g/ml cycloheximide. No difference in degradation rate was noted between cells incubated with or without GH. LAP, liver-enriched transcriptional activator protein; LIP, liver-enriched transcriptional inhibitory protein. Statistically significant differences were: * $P < 0.05$ and $^{\dagger}P < 0.01$.

Figure 3 GH facilitates the nuclear translocation of Stat5. 3T3-L1 cells were incubated with differentiation medium for 3 h followed by incubation with maintenance medium, and 50 ng/ml GH were added to both differentiation and maintenance media. (A) Lysates from cytosol extracts and nuclear extracts were prepared at the indicated periods and then subjected to western blot analysis of Stat5, β -actin (cytosol extracts), and PCNA (nuclear extracts). (B) The protein expression of nuclear Stat5 was quantified by densitometry from three independent experiments. Densities were normalized against the density of PCNA. Statistically significant differences were: * $P < 0.001$, $^{\dagger}P < 0.01$, and $^{\ddagger}P < 0.05$.

medium, was reported to activate Stat5 (Ruff-Jamison *et al.* 1995). Combined together, nuclear Stat5 expression might be regulated by the combination of GH-dependent activation in the early stage of adipogenesis and GH-independent activation in the later stage of adipogenesis. Although the exact reason why levels of nuclear Stat5 fluctuate in this way in cells with GH and the outcome of this fluctuation are not clear, GH is thought to function at an early stage of adipogenesis. In conclusion, GH facilitates the nuclear translocation of Stat5 and the GH-Stat5 signaling pathway is possibly involved in the stimulatory effect of GH in the early stage of adipogenesis.

Stat5A-Y694F suppresses the stimulatory effect of GH in MDI-induced adipogenesis, while Stat5A and 5B enhance MDI-induced adipogenesis

In order to examine whether the adipogenic effect of GH is mediated through Stat5A and/or 5B, we used a point mutant of Stat5A (Stat5A-Y694F; Nanbu-Wakao

et al. 2002). First, we examined the effect of Stat5A-Y694F on the transcriptional activity of Stat5A and 5B in 3T3-L1 cells by a luciferase reporter assay using a β -casein gene promoter luciferase construct containing a Stat5 response element. In the presence of GH, Stat5A and 5B showed 21- and 7-fold enhancement of transcriptional activity respectively. Co-expression of Stat5A-Y694F showed approximately 80 and 70% suppression of the transcriptional activity of Stat5A and 5B respectively, thus indicating that Stat5A-Y694F functions as a transcriptional suppressor for both the GH-Stat5A and the GH-Stat5B signaling pathways in 3T3-L1 cells (Fig. 4A).

Next, we explored the effect of overexpression of Stat5A-Y694F, Stat5A, and Stat5B in the adipogenic effect of GH using adenovirus gene transfer system. Two days after infection, the cells were induced to differentiate by 3-h incubation with differentiation medium followed by the incubation with maintenance medium, and GH was added to both differentiation and

Figure 4 Involvement of the GH-Stat5A and the GH-Stat5B signaling pathway in MDI-induced adipogenesis. (A) Overexpression of Stat5A-Y694F suppressed the transcriptional activity of Stat5A and 5B. 3T3-L1 cells were transfected with pZZ1 (0.1 μg), TK-*Renilla* reporter plasmid (5 ng), GH receptor expression vector (0.2 μg), Stat5A or 5B expression vector (0.05 μg), and Stat5A-Y694F expression vector (0.2 μg). The cells were treated with or without 500 ng/ml GH in DMEM for 16 h. Luciferase activity was measured using cell extracts and normalized by determining *Renilla* luciferase activity. Experiments were repeated independently five times. (B-E) Stat5A-Y694F suppressed the stimulatory effect of GH with the reduction of PPAR $\gamma 2$ expression, while Stat5A and 5B enhanced MDI-induced adipogenesis with the enhancement of PPAR $\gamma 2$ expression. 3T3-L1 cells were infected with LacZ, Stat5A, 5B, or Stat5A-Y694F adenovirus. After 2 days, the cells were induced to differentiate with differentiation medium for 3 h followed by incubation with maintenance medium, and 50 ng/ml GH were added to both differentiation and maintenance media. On day 6, the cells were stained with Oil Red O (B) and the stained area was measured (C). Whole cell lysates were collected 9 h after adipogenic induction and then subjected to western blot analysis of PPAR γ , Stat5, and β -actin (D). The protein expression of PPAR $\gamma 2$ was quantified by densitometry from three independent experiments. Densities were normalized against the density of β -actin (E). Statistically significant differences were: * $P < 0.0001$, $^{\dagger}P < 0.01$, and $^{\ddagger}P < 0.05$.

maintenance media. Overexpression of Stat5A-Y694F significantly suppresses the stimulatory effect of GH during MDI-induced adipogenesis with reduction of PPAR γ 2 expression (Fig. 4B–E), indicating the involvement of the GH-Stat5A/5B signaling pathway in GH-mediated adipogenesis with modulation of PPAR γ expression. In addition, western blot analysis of PPAR γ 9 h after induction showed increased PPAR γ 2 expression in the cells infected with Stat5A or 5B in a GH-dependent manner (Fig. 4D and E). Oil Red O staining at day 6 showed that overexpression of Stat5A or 5B stimulated MDI-induced adipogenesis, however, GH-dependent manner was not noted (Fig. 4B and C).

Stat5A/5B stimulates C/EBP β - and C/EBP δ -induced adipogenesis

Since PPAR γ is induced by C/EBP β and C/EBP δ during adipogenesis, we examined the relationship between Stat5A/5B and C/EBP β / δ . Overexpression of Stat5A or 5B in 3T3-L1 cells did not induce adipogenesis, indicating that Stat5A/5B by itself has little effect on the initiation of adipogenesis (Fig. 5A). When C/EBP β or C/EBP δ was overexpressed in 3T3-L1 cells and adipogenic program was initiated, co-expression of Stat5A or 5B further enhanced C/EBP β - and C/EBP δ -induced adipogenesis (Fig. 5A and B). Consistent with this result, Stat5A and 5B enhanced PPAR γ 2 expression in 3T3-L1 cells induced by C/EBP β or C/EBP δ (Fig. 5C and D). These findings demonstrate that Stat5A/5B is involved in adipogenesis in cooperation with C/EBP β / δ . To support this notion, we investigated the effect of Stat5A and 5B on the transcriptional activity of C/EBP β / δ in the PPAR γ gene promoter in 3T3-L1 cells. Overexpression of Stat5A and 5B showed 145 ± 16 and $118 \pm 7\%$ enhancement of C/EBP β / δ transcriptional activity respectively (Fig. 5E).

Stat5A/5B stimulates adipogenesis in cooperation with PPAR γ

Although Stat5A/5B enhanced PPAR γ expression in C/EBP β - and C/EBP δ -induced adipogenesis of 3T3-L1 cells, this does not exclude the possibility that Stat5A/5B has any effects on PPAR γ activity. To examine this, we overexpressed PPAR γ in 3T3-L1 cells. Overexpression of PPAR γ induced adipogenesis, and co-expression of Stat5A or 5B stimulated PPAR γ -induced adipogenesis (Fig. 6A–C). To further confirm whether Stat5A/5B have any effects on PPAR γ activity, a luciferase reporter assay using PPAR γ -binding element (PPRE)-luciferase construct was performed. The GH-Stat5A and the GH-Stat5B signaling pathways showed the trend of enhancement of the transcriptional activity of PPAR γ in 3T3-L1 cells (Fig. 6D). However, due to the

large variation in the data, there was no significant difference. We speculated that this outcome might be due to low transfection efficiency in 3T3-L1 cells. Therefore, to add further evidence, we investigated this effect using a multipotent mesenchymal cell line, C3H10T1/2, in which high transfection efficiency was achieved. In C3H10T1/2 cells, overexpression of Stat5A or 5B also stimulated PPAR γ -induced adipogenesis and the GH-Stat5A and the GH-Stat5B signaling pathways significantly enhanced the transcriptional activity of PPAR γ by 145 ± 10 and $191 \pm 3\%$ in the presence of TGZ respectively (Fig. 6 A, B, and D). These findings indicate that Stat5A/5B functions by enhancing the transcriptional activity of PPAR γ .

cDNA microarray analysis

Although Stat5A/5B has been reported to have an important role in adipose tissue formation, and our data suggest that GH is likely to exert its effect through Stat5A/5B, there are possibly other pathways through which GH exerts its effect on adipogenesis. In fact, some genes have been reported to be induced in response to GH during adipogenesis in 3T3-F442A cells (Gurland *et al.* 1990, Clarkson *et al.* 1995, 1999, Shang *et al.* 2002, Huo *et al.* 2006). To investigate the mechanisms by which GH stimulates adipogenesis, we analyzed the gene expression profiles of 3T3-L1 cells treated with GH during MDI-induced adipogenesis using cDNA microarray analysis. Adipogenesis was induced with the incubation of differentiation medium in the presence or absence of GH, and total RNA was extracted 3 h after adipogenic induction. As we intended to detect the genes that affect PPAR γ expression, we set the point of RNA extraction as 3 h after induction when PPAR γ 2 expression was not detected by western blot analysis. Gene expression was compared between cells treated with or without GH. cDNA microarray analysis was performed thrice, and genes which showed an increment or decrement of more than 1.5-fold in the fluorescence level at least twice were selected as up- and down-regulated genes respectively. The number of up- and down-regulated genes was 18 and 19 among the 4277 spotted genes respectively (Tables 1 and 2). Among the genes associated with adipogenesis, PPAR γ was up-regulated, supporting our findings that GH induces PPAR γ expression during adipogenesis.

In addition, the expression of genes coding for growth factors or growth factor receptors was altered. Among them, mRNA levels of IL-4 receptor, VEGF-A, and TWIST were confirmed using semiquantitative RT-PCR analysis (Fig. 7). Since the expression of these genes was altered by MDI-treatment alone, GH is thought to function under the influence of MDI-treatment. So far, we have not been able to identify

Figure 5 Stimulation of C/EBP β - or C/EBP δ -induced adipogenesis by Stat5A and 5B. 3T3-L1 cells were infected with LacZ, C/EBP β or C/EBP δ adenovirus together with Stat5A or 5B adenovirus, and cultured for 6 days in DMEM containing 10% FCS. (A and B) The cells were stained with Oil Red O (A) and the stained area was measured (B). (C and D) Whole cell lysates were collected and subjected to western blot analysis of PPAR γ , C/EBP β , C/EBP δ , Stat5, and β -actin (C). The protein expression of PPAR γ 2 was quantified by densitometry from three independent experiments. Densities were normalized against the density of β -actin (D). (E) Enhancement of the transcriptional activity of C/EBP β / δ in the PPAR γ gene promoter by Stat5A and 5B. 3T3-L1 cells were transfected with PPAR γ gene promoter luciferase construct (0.1 μ g), TK-*Renilla* reporter plasmid (5 ng), GH receptor expression vector (0.2 μ g), C/EBP β expression vector (0.025 μ g), C/EBP δ expression vector (0.025 μ g), and Stat5A or 5B expression vector (0.05 μ g). The cells were treated with 500 ng/ml GH in DMEM for 16 h. Luciferase activity was measured using cell extracts and normalized by determining *Renilla* luciferase activity. Experiments were repeated independently seven times. Statistically significant differences were: * $P < 0.01$ and $^{\dagger}P < 0.05$.

Figure 6 Stat5A and 5B stimulate adipogenesis in association with PPAR γ . (A–C) 3T3-L1 cells and C3H10T1/2 cells were infected with PPAR γ adenovirus together with or without Stat5A or 5B adenovirus and cultured for 6 days in DMEM containing 10% FCS. The cells were stained with Oil Red O (A) and the stained area was measured (B). Whole cell lysates were collected and subjected to western blot analysis of PPAR γ , STAT5, and β -actin (C). (D) Stat5A and 5B enhance the transcriptional activity of PPAR γ . 3T3-L1 cells and C3H10T1/2 cells were transfected with 3 $\times$ PPRE-Luc (0.1 μ g), TK-*Renilla* reporter plasmid (5 ng), GH receptor expression vector (0.2 μ g), Stat5A or 5B expression vector (0.05 μ g), and PPAR γ expression vector (0.05 μ g). The cells were treated with either 500 ng/ml GH or 1 μ M troglitazone (TGZ), or both in DMEM for 16 h. Luciferase activity was measured using cell extracts and normalized by determining *Renilla* luciferase activity. Experiments were repeated independently five times. Statistically significant differences were: * $P < 0.0001$, [†] $P < 0.001$, [‡] $P < 0.01$, and [§] $P < 0.05$. ns, not significant.

Table 1 Up-regulated genes

Gene	Folds			Function	
	1st	2nd	3rd		
Accession no.					
NM_007836	Growth arrest and DNA damage-inducible 45	2.7717	3.0877	2.0545	Cell cycle
NM_026179	RIKEN cDNA 1300003D03 gene	2.3945	3.0000	2.3114	Unknown
NM_021434	Hypothetical protein, MNCb-3029	2.0402	1.5520	2.0516	Unknown
NM_023118	Disabled homolog 2 (<i>Drosophila</i>)	2.1554	1.7371	1.5896	Cell development
NM_010557	Interleukin 4 receptor, α	1.8548	1.7473	1.8151	Signal transducer
NM_011035	p21 (CDKN1A)-activated kinase 1	1.8396	1.4252	1.9949	Cell development
NM_011106	Protein kinase inhibitor, γ	1.6938	1.6333	1.8828	Enzyme
NM_011198	Prostaglandin-endoperoxide synthase 2	1.7094	1.4717	1.6432	Enzyme
NM_009505	Vascular endothelial growth factor A	1.5886	1.5448	1.6354	Growth factor
NM_011521	Syndecan 4	1.7141	1.3924	1.5432	Extracellular matrix protein
NM_009178	Sialyltransferase 4C (β -galactosidase α -2,3-sialyltransferase)	1.4699	1.6359	1.5141	Enzyme
NM_139198	Placenta-specific 8	1.4344	1.5240	1.5624	Unknown
NM_018884	semaF cytoplasmic domain associated protein 3	1.3547	1.5022	1.6223	Cell communication
NM_020573	Oxysterol-binding protein-like 1A	1.4203	1.5253	1.5297	Signal transducer
NM_011660	Thioredoxin 1	1.5666	1.2777	1.5973	Electron transport
NM_011146	Peroxisome proliferator activated receptor γ	1.6205	1.1918	1.6263	Transcription factor
NM_010135	Enabled homolog (<i>Drosophila</i>)	1.5970	1.1987	1.5431	Cytoskeleton organization
NM_009846	CD24a antigen	1.1468	1.5269	1.5196	Cell surface protein

the genes that are regulated by GH independent of MDI-treatment during GH-mediated adipogenesis.

Discussion

Consistent with the clinical features of GHD patients, several reports have suggested a stimulatory effect of GH on adipogenesis (Morikawa *et al.* 1984, Nixon & Green 1984). While GHD patients show a decreased

number of adipocytes, they can form adipose tissue (Bonnet *et al.* 1974, Salomon *et al.* 1989, Bengtsson *et al.* 1993). These findings suggest that GH is not a major regulator, but an important modulator for adipogenesis. In this study, GH alone could not induce adipogenesis of 3T3-L1 cells. However, GH could stimulate adipogenesis when agents with adipogenic action, such as MDI, were added. These findings support the suggestion that GH stimulates adipogenesis by modulating the adipogenic program. In contrast to

Table 2 Down-regulated genes

		Folds			
Gene		1st	2nd	3rd	Function
Accession no.					
NM_020578	EH-domain containing 3	0-5780	0-4894	0-5072	Endocytic vesicle
NM_011315	Serum amyloid A 3	0-4740	0-5489	0-5852	Defense/immunity protein
NM_011693	Vascular cell adhesion molecule 1	0-5901	0-5508	0-5181	Cell adhesion
NM_011588	Tripartite motif protein 28	0-6063	0-6823	0-5549	Transcription
NM_013928	Schwannomin interacting protein 1	0-5723	0-6601	0-6173	Cytoplasmic protein
NM_009621	A disintegrin-like and metalloprotease (reprolysin type)	0-6421	0-6600	0-5714	Enzyme
NM_011058	Platelet-derived growth factor receptor, α polypeptide	0-5712	0-7004	0-6283	Signal transducer
NM_030705	Mesoderm development candidate 1	0-6756	0-6864	0-6099	Unknown
NM_010863	Mouse myosin Ib	0-7174	0-6706	0-5844	Motor protein
NM_020581	Angiopoietin-like 4	0-6290	0-7032	0-6454	Unknown
NM_008008	Fibroblast growth factor 7	0-6805	0-6436	0-6657	Growth factor
NM_009372	TG interacting factor	0-7256	0-6667	0-6213	Transcription
NM_013598	kit ligand	0-7613	0-6511	0-6289	Signal transducer
NM_011658	Twist gene homolog, (<i>Drosophila</i>)	0-7358	0-6736	0-6507	Transcription
NM_007672	Cerebellar degeneration-related 2	0-8974	0-5588	0-6178	Unknown
NM_007913	Early growth response 1	0-6580	0-6558	0-7774	Transcription
NM_019512	Transcription elongation regulator 1 (CA150)	1-0955	0-3478	0-6659	Transcription
NM_009640	Angiopoietin 1	0-8618	0-6105	0-6460	Signal transducer
NM_017478	Coatomer protein complex, subunit γ 2	0-8699	0-6667	0-6331	Cytoplasmic vesicle

Figure 7 Semiquantitative RT-PCR analysis of IL-4 receptor, VEGF-A, and TWIST. 3T3-L1 cells were induced to differentiate with 3-h treatment of differentiation medium. Total RNA was isolated 3 h after adipogenic induction and cDNA was synthesized. IL-4 receptor and VEGF-A were up-regulated and TWIST was down-regulated in cells treated with GH.

the data from cell lines, an inhibitory effect of GH on adipogenesis was reported in primary preadipocytes (Wabitsch *et al.* 1996, Hansen *et al.* 1998, Richter *et al.* 2003). This conflicting result of GH action may come from the different characteristics between cell lines and primary preadipocytes. Actually, it was pointed out that primary preadipocytes may have been at a later stage of adipogenesis beyond that of established preadipocyte models (Wabitsch *et al.* 1995, Gregoire *et al.* 1998, Nam & Marcus 2000). Alternatively, treatment with GH is not required in primary cultures due to their prior *in vivo* exposure to circulating GH. We speculate that primary preadipocytes have already been primed with GH and therefore GH cannot stimulate adipogenesis in primary preadipocytes. To clarify the role of GH on adipogenesis more clearly, we think it useful to use the primary preadipocytes isolated from GHD mice (*lit/lit* mice).

In cells subject to our differentiation protocol, the expression of PPAR γ 2 increased earlier in cells treated with GH. Although the enhancement of C/EBP β and/or C/EBP δ expression was thought to be a candidate to induce the early expression of PPAR γ 2, GH did not have any influence on the expression of C/EBP β and C/EBP δ in 3T3-L1 cells. Since we cannot exclude the possibility of involvement of C/EBP β and/or C/EBP δ in GH-mediated adipogenesis in 3T3-L1 cells, the intracellular localization of C/EBP β and C/EBP δ during GH-mediated adipogenesis was assessed by immunofluorescence taken 1 h after induction. Expression of C/EBP β and C/EBP δ was restricted in the nucleus and there was no difference between the cells treated with or without GH (data not shown). Furthermore, GH was reported to cause the rapid and transient dephosphorylation of C/EBP β in 3T3-F442A cells (Liao *et al.* 1999, Piwien-Pilipuk *et al.* 2001). Therefore, we analyzed the dephosphorylation

of C/EBP β in response to GH in 3T3-L1 cells during adipogenesis. Lysates from nuclear extracts were separated with SDS-PAGE and immunoblotted with C/EBP β antibody, showing no band representing the faster migrating forms of C/EBP β indicated in the previous reports (Liao *et al.* 1999, Piwien-Pilipuk *et al.* 2001; data not shown). Our data, together with previous studies, demonstrated that GH stimulates adipogenesis of 3T3-L1 cells through the enhancement of PPAR γ expression, not C/EBP β / δ expression (Tominaga *et al.* 2002). However, the mechanisms by which GH enhances PPAR γ expression remain to be determined.

GH mediates its action through various pathways, such as Stat, mitogen activated protein kinase (MAPK), and phosphatidylinositol 3 kinase (PI3K) (Piwien-Pilipuk *et al.* 2002). Among these signaling molecules, Stat5 was reported to be involved in adipose tissue formation (Teglund *et al.* 1998). Therefore, we examined the effect of Stat5A/5B on GH-mediated adipogenesis. To investigate the role of Stat5A/5B in the adipogenic effect of GH, Stat5A-Y694F was utilized. Overexpression of Stat5A-Y694F suppressed the stimulatory effect of GH in MDI-induced adipogenesis with the reduction of PPAR γ 2 expression, indicating that GH mediates its adipogenic effect through Stat5A/5B PPAR γ . However, the stimulatory effect of GH was not completely diminished by overexpression of Stat5A-Y694F. This is probably because remaining endogenous Stat5A/5B activity mediates some GH action and/or because factors other than Stat5A/5B may be involved in this stimulatory effect of GH. In addition, overexpression of Stat5A and 5B enhanced MDI-induced adipogenesis. Although PPAR γ 2 expression at an early stage of adipogenesis was enhanced in a GH-dependent manner, Oil Red O staining 6 days after induction showed no GH-dependent enhancement (Fig. 4C). This finding was partly explained by the Stat5 activation independent of GH over 24 h after adipogenic induction (Fig. 3A and B). Western blot analysis in Fig. 4D and E was performed 9 h after adipogenic induction, when the cells in the absence of GH showed no nuclear translocation of Stat5. In this condition, GH significantly induced the PPAR γ 2 expression. Therefore, the up-regulation of PPAR γ 2 in the cells with GH could be explained by the difference of nuclear Stat5 expression. While Oil Red Staining in Fig. 4B and C was performed 6 days after induction, Stat5 is translocated into nucleus even in the cells without GH. GH-independent activation of Stat5, especially in the culture system where Stat5 is overexpressed, may have been enough to stimulate MDI-induced adipogenesis even in the absence of GH.

Although Stat5A is reported to be necessary for GH-dependent adipogenesis in 3T3-F442A cells (Yarwood *et al.* 1999, Shang & Waters 2003), as far as we know, our study is the first report investigating the

adipogenic effect of the GH-Stat5A signaling pathway in 3T3-L1 cells. Nanbu-Wakao *et al.* (2002) reported that the overexpression of Stat5A stimulates adipogenesis and Stat5A-Y694F partially suppresses adipogenesis of 3T3-L1 cells, and Stewart *et al.* (2004) reported that Stat5 activator can replace the requirement of FCS in 3T3-L1 adipogenesis. However, the effect of GH on adipogenesis was not demonstrated in these reports. In addition to the adipogenic effect of the GH-Stat5A signaling pathway, we also revealed the adipogenic effect of the GH-Stat5B signaling pathway. As Floyd & Stephens (2003) reported that Stat5B cannot stimulate adipogenesis in both BALB/c and NIH-3T3 cells treated with MDI, this difference may come from the use of different cells.

Importantly, our findings also suggested the possibility that PPAR γ mediates the adipogenic effect of the GH-Stat5A/5B signaling pathway. To elucidate the role of PPAR γ in the adipogenic effect of the GH-Stat5A/B signaling pathway, we first investigated the relationship between Stat5A/5B and C/EBP β / δ . In the present study, Stat5A and 5B stimulated C/EBP β - and C/EBP δ -induced adipogenesis with the enhancement of PPAR γ expression. Since Stat5A and 5B enhanced the transcriptional activity of C/EBP β / δ in the PPAR γ gene promoter, this suggests that Stat5A and 5B stimulate adipogenesis by inducing PPAR γ expression in cooperation with C/EBP β / δ . Next, we investigated the relationship between Stat5A/5B and PPAR γ . Stat5A and 5B stimulated PPAR γ -induced adipogenesis and enhanced the transcriptional activity of PPAR γ , indicating that Stat5A and 5B stimulate adipogenesis by reinforcing the transcriptional activity of PPAR γ . In contrast to our results, Stat5A and 5B have been reported to inhibit the transcriptional activity of PPAR γ in COS1 and HEK293 cells (Richter *et al.* 2003, Shipley & Waxman 2004). These results were repeatable in our hands (data not shown). These findings indicate that the effect of Stat5A/5B on the transcriptional activity of PPAR γ is dependent on the cell type and Stat5A/5B up-regulates the transcriptional activity of PPAR γ in cell lines with adipogenic potential, including 3T3-L1 and C3H10T1/2 cells. Taken together, the GH-Stat5A/5B signaling pathway stimulates adipogenesis through two distinct steps; one is transcriptional activation of C/EBP β / δ in the PPAR γ gene promoter and the other is direct activation of PPAR γ activity.

Although we mainly focused on the postreceptor events in this study, it is important to consider the role of GHR to completely understand the role of GH. GHR has the truncated splice variants, which inhibit full-length GHR in a dominant-negative manner (Ross *et al.* 1997). In 3T3-L1 cells, full-length GHR is expressed 100 times as much as the truncated form of GHR, and expression of full-length GHR increases as adipogenesis proceeds, while the truncated form does not (Iida *et al.*

2003). We performed RT-PCR analysis and obtained similar findings (data not shown). Considering the abundance of full-length GHR expression and the finding that expression level of full-length GHR increases in parallel with adipogenesis, dominant-negative effect of truncated variants of GHR has minimal effect of GH-mediated adipogenesis in 3T3-L1 cells. We have to consider the effect of GH-binding protein (GHBP), because expression of GHBP increases in parallel with adipogenesis (Zou *et al.* 1997). GHBP has been demonstrated to compete with GHR for ligand binding in the extracellular space *in vitro* (Lim *et al.* 1990); however, other aspects of GHBP function have been reported (Bick *et al.* 1994, Graichen *et al.* 2003). GHBP is also localized intracellularly, both attached to intracellular membranes and soluble in the cytoplasm and nucleoplasm (Lobie *et al.* 1992, Frick *et al.* 1994), and intracellular GHBP functions as a transcriptional enhancer in GH-stimulated Stat5-mediated transcription (Graichen *et al.* 2003). Therefore, we cannot exclude the possibility that GH stimulates adipogenesis partly through intracellular GHBP and further investigations are needed to clarify this.

Other genes have also been reported to be involved in the adipogenic effect of GH (Clarkson *et al.* 1999, Shang *et al.* 2002). IGF-I has been reported to stimulate adipogenesis of 3T3-L1 cells (Smith *et al.* 1988). As the production of IGF-I is up-regulated in response to GH in various cells (Piwien-Pilipuk *et al.* 2002), there arises the possibility that GH exerts its adipogenic effect through production of IGF-I. To address this, we examined IGF-I expression by semiquantitative RT-PCR analysis. IGF-I expression could not be detected in 3T3-L1 preadipocytes, while it was detected in 3T3-L1 adipocytes (data not shown). When analyzed 3 h after adipogenic induction, IGF-I expression was not detected in cells treated with or without GH (data not shown). These results indicate the low possibility of IGF-I involvement in the stimulatory effect of GH in the early stage of adipogenesis.

Next, we analyzed the expression of genes at an early stage using cDNA microarray analysis (Tables 1 and 2). The expression of IL-4 receptor transiently increases during the early stage of adipogenesis of 3T3-L1 cells treated with MDI, and the expression pattern of IL-4 receptor was reported to be very similar to that of C/EBP β (Hua *et al.* 2004). Our cDNA microarray analysis demonstrated that the expression of IL-4 receptor increased in cells treated with GH, suggesting the possibility that IL-4 signaling participates in the adipogenic effect of GH. Considering the expression pattern of IL-4 receptor, IL-4 signaling may be involved in clonal expansion. However, no proof of adipogenic effect of IL-4 signaling was reported (Hua *et al.* 2004).

VEGF-A expression was also up-regulated in cells treated with GH. Adipose tissue mass can be regulated by its vasculature (Rupnick *et al.* 2002). In addition, reciprocal interaction between adipogenesis and angiogenesis is crucial in the formation of adipose tissue, and VEGF-A was reported to be involved in this interaction (Fukumura *et al.* 2003). VEGF-A is expressed in adipocytes but not in preadipocytes both *in vitro* and *in vivo* (Soukas *et al.* 2001). As GH is important in the regulation of angiogenesis, it may induce adipogenesis through the promotion of angiogenesis. Other than VEGF-A, the expression levels of growth factors and their receptors were altered in response to GH. Although their contribution to adipogenesis was unclear, these results suggest that GH stimulates adipogenesis in association with cell growth modulation. In addition to growth factors, the expression of TWIST, a basic helix-loop-helix transcription factor, was down-regulated. The down-regulation of TWIST may have an impact on adipogenesis because TWIST is involved in osteogenesis and chondrogenesis (Bialek *et al.* 2004, Reinhold *et al.* 2006), and adipocytes, osteoblasts, and chondrocytes originate from the same precursor stem cells, mesenchymal stem cells.

In summary, our data demonstrate that GH stimulates adipogenesis under adipogenic induction through the enhancement of PPAR γ expression, and both Stat5A and 5B are involved in the adipogenic effect of GH. In addition, we provide evidence that Stat5A and 5B function in association with C/EBP β , C/EBP δ , and PPAR γ resulting in the promotion of adipogenesis.

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