Identification and characterization of NYGGF4, a novel gene containing a phosphotyrosine-binding (PTB) domain that stimulates 3T3-L1 preadipocytes proliferation

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Received 15 March 2006; received in revised form 1 May 2006; accepted 4 May 2006
Available online 25 May 2006
Received by J.A. Engler

Abstract

A novel gene named NYGGF4, which was expressed at a higher level in obese subjects, was isolated and characterized. It is a 1527-bp cDNA, containing 753 nucleotides of an ORF (open reading frame) predicting 250 amino acids with a molecular mass of 28.27 kDa. Amino acid sequence analysis revealed NYGGF4 has a phosphotyrosine-binding (PTB) domain. Northern blot analysis revealed NYGGF4 is expressed primarily in adipose tissue, heart, and skeletal muscle but not in any other tissue examined. Confocal imagery analyses with green fluorescent protein-tagged protein transiently expressed in 3T3-L1 preadipocytes and 293-T cells show that NYGGF4 localizes in the cytoplasm. Furthermore, ectopic expression of NYGGF4 dramatically increases the proliferation of 3T3-L1 preadipocytes without affecting adipocytic differentiation. And the NYGGF4 expression 3T3-L1 cells had a greater number of cells in S-phase. Our data suggest that NYGGF4 might be a signaling molecule and could play a role in cell growth and adipogenesis process.

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Keywords: Suppression subtractive hybridization; Tissue distribution; Intracellular localization; Cell cycle; 3T3-L1 differentiation; Obesity

1. Introduction

Obesity is a multifactorial disease resulting from the interactions between susceptibility genes and environmental factors. It is an important risk factor for various diseases such as type 2 diabetes, hypertension, hyperlipidemia and cardiac infarction (Spiegelman and Flier, 2001; Kahn and Flier, 2000; Visscher and Seidell, 2001). During the development of obesity, adipose tissue plays a key role in energy homeostasis by regulating the balance between energy storage and release according to nutritional status (Spiegelman and Flier, 2001). Adipocytes synthesize and store triglyceride in periods of nutritional abundance and mobilize the lipids in response to fasting (Spiegelman et al., 1993; Spiegelman and Hotamisligil, 1993). Fat tissue is also involved in regulating blood glucose levels through the expression of the insulin responsive glucose transporter, Glut4 (Kaestner et al., 1989, 1990). Now it is recognized that adipose tissue is a major secretory organ that secretes numerous kinds of cytokines including tumor necrosis factor-alpha, leptin, adiponectin and resistin. These factors are known to influence insulin sensitivity, food intake, arteriosclerosis, and several common diseases (Spiegelman and Flier, 1996; Maeda et al., 2002). Thus, identifying some of the genes differentially expressed in adipose tissue between obese and normal

Abbreviations: A, absorbance; bp, base pair(s); BMI, body mass index; cDNA, DNA complementary to RNA; DMEM, Dulbecco’s Modified Eagle Medium; FBS, fetal bovine serum; G418, Geneticin; GFP, green fluorescent protein; GLUT-4, Glucose Transporter-4; His, histidine; kb, kilobase(s); kDa, kilodalton(s); ORF, open reading frame; PCR, polymerase chain reaction; PTB, phosphotyrosine-binding; UV, ultraviolet.

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0378-1119/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2006.05.008
individuals could potentially be related to the regulation of body mass and to the pathogenesis of obesity. The identification of such genes may provide new candidates for ongoing and future studies.

To help clarify the mechanism for obesity development, mRNAs expressed in abdominal subcutaneous fat from obese subjects (BMI > 30 kg/m²) were subtracted with those from normal-weight subjects (BMI = 18 – 25 kg/m²) using suppression subtractive hybridization (SSH) (Diatchenko et al., 1996, 1999).

We cloned a novel cDNA that was expressed at a higher level in obese subjects and named as NYGGF4 (Genbank Accession No. AY317148). The 1527-bp cDNA contains 753 nucleotides of an ORF predicting 250 amino acids with a molecular mass of 28.27 kDa. Using a domain predicting program, the predicted NYGGF4 protein was shown to contain a phosphotyrosine-binding (PTB) domain. The PTB domain was originally identified as a 186-residue segment of the signaling protein Shc and binds specifically to the tyrosine-phosphorylated form of an unidentified 145-kD protein in response to many growth factors (Kavanaugh and Williams, 1994). This indicates that NYGGF4 may encode a signaling molecule and play a role in cell growth. No more can be deduced from its sequence.

In this study, we report the cDNA and deduced amino acid sequence of the new gene, NYGGF4, its tissue expression patterns and its subcellular localization. Moreover, to characterize its potential role in cell growth we stably transfected NYGGF4 into preadipocyte 3T3-L1 cells and found it dramatically increased the growth rate of the 3T3-L1 cells without affecting adipocytic differentiation.

2. Materials and methods

2.1. Human subjects

Written consent was obtained from each subject and experiments were conducted according to the Declaration of Helsinki. Subcutaneous abdominal adipose tissue was obtained by direct acquisition from individuals undergoing abdominal surgery for appendicitis.

2.2. Suppression subtractive hybridization (SSH)

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) from subcutaneous abdominal adipose tissue and Polyadenylated (PolyA+) mRNA was subsequently isolated using a NucleoTrap® Nucleic Acid Purification Kit (Clontech, Mountain View, CA). SSH was performed with the PCR-selectTM cDNA Subtractive Kit (Clontech, Mountain View, CA) according to the manufacturer’s instructions. Two directional subtractions were carried out. In the forward (Ob-specific) subtraction, the genes specifically expressed or highly expressed in subcutaneous abdominal adipose tissue from obese subjects were identified. The reverse (N-specific) subtraction was conducted to analyze the genes predominantly expressed in normal subjects. A control subtraction was also performed to estimate the subtractive efficiency. Thereafter, PCR products from SSH were directly cloned into pGEM®-T Easy Vector (Promega, Madison, WI) and transformed into E. coli strain XL-1’ Blue supercompetent cells (Stratagene, La Jolla, CA).

2.3. Differential expression

Confirmation of NYGGF4 differential expression was made by RT-PCR. Total RNA was extracted from subcutaneous abdominal adipose tissue of six obese (BMI > 30 kg/m²) and six normal-weight (BMI from 18 kg/m² to 25 kg/m²) subjects, whom were randomly selected from the two groups. Samples of 200 ng of total RNA were subjected to reverse transcription using random primers with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and an aliquot (10%) of the resulting cDNA was amplified with GAPDH and NYGGF4 gene-specific primer (sequences given in Table 1). After 3 min at 94 °C for denaturation, PCR was performed for twenty-eight cycles of 30 s at 94 °C, 30 s at 58 °C and 40 s at 72 °C. A final extension step was performed at 72 °C for 7 min. Five microliters of PCR products was loaded on a 1.5% agarose gel.

2.4. Northern blot analysis

Total RNA was isolated from human subcutaneous abdominal adipose tissue with TRIzol (Invitrogen, Carlsbad, CA). Twenty micrograms of total RNA was electrophoresed in a 1% agarose gel containing 2% formaldehyde, then transferred to Hybond-N nylon membrane (Clontech, Mountain View, CA) and UV-cross-linked. For analysis of NYGGF4 expression in human different tissues, a human multiple tissue Northern blot was purchased from Clontech (Mountain View, CA). 32P-labeled probes were synthesized according to the open reading fragment of human NYGGF4 by the random primer method (Feinberg and Vogelstein, 1983). After hybridization in ExpressHyb solution (Clontech, Mountain View, CA) at 42 °C for 16 h and sequential washing, the membranes were exposed to X-ray film at −80 °C for three days.

Table 1

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Product size (bp)</th>
<th>Reverse and forward primer (5’–3’)</th>
<th>T_a (°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>NYGGF4</td>
<td>750</td>
<td>R: GCCATACGGGATCTCAATTC F: ATGTCAGCCTGCGCTTAG</td>
<td>58</td>
<td>28</td>
</tr>
<tr>
<td>GAPDH</td>
<td>443</td>
<td>R: TGACCTTGCACAGCCTTG F: CATCACATCTCCAGGAGGG</td>
<td>58</td>
<td>28</td>
</tr>
<tr>
<td>Mouse PPARγ</td>
<td>266</td>
<td>R: GCCACACTGGATCTCTTTL F: CCAAGAACGTGGCCACTCA</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td>Mouse PPARγ</td>
<td>582</td>
<td>R: GAGGCTTGTGCTCCAGTGGAA F: CCAAGAAGTGTTCGCACTGT</td>
<td>56</td>
<td>32</td>
</tr>
<tr>
<td>Mouse AP2</td>
<td>480</td>
<td>R: TCTCCTGATGACGCTGAT F: GTGTTCTCTGTCACCTC</td>
<td>58</td>
<td>29</td>
</tr>
</tbody>
</table>

PPARγ, peroxisome proliferator-activated receptor gamma; AP2, adipocyte lipid binding protein gene; LPL, lipoprotein lipase; R: reverse primer; F: forward primer; T_a: annealing temperature.
2.5. Intracellular localization of NYGGF4

Plasmid pEGFP-N2-NYGGF4, was constructed by sub cloning the NYGGF4 PCR-generated coding sequence fragment into the HindIII and the KpnI sites of pEGFP-N2 vector with a pair of primers 5′-CCC AAG CTT ATG TTC AGC CTG-3′ and 5′-CGG GAA TTC CAG CCA TCA TCG GA-3′ to generate a plasmid expressing NYGGF4-6×His fusion protein. Expression vectors carrying the NYGGF4 coding sequence or empty vectors were transfected into 3T3-L1 cells. Two days after transfection, the cells were plated onto cover slips placed in six-well culture plates for live cell microscopy and cultured for 24 h. Then the cells were transfected using Fugene™ 6 transfection reagent (Roche, Basel, Switzerland). At 24 h post-transfection, cells were observed for GFP expression using a confocal microscope.

2.6. Establishment of NYGGF4-expressing transformants

The coding sequence of NYGGF4 was subcloned into the HindIII and the EcoRI sites of pcDNA3.1Myc/His (B) vector using oligonucleotides 5′-CCC AAG CTT CTG TTC AGC CTG CCC-3′ and 5′-CCG GAA TTC CAG CCA TCA TCG GA-3′ to generate a plasmid expressing NYGGF4-6×His fusion protein. Expression vectors carrying the NYGGF4 coding sequence or empty vectors were transfected into 3T3-L1 cells. Two days after transfection, neomycin (G418, Roche, Basel, Switzerland) was added to the medium (800 μg/ml) to select for transfected cells. Drug-resistant cells began to form small colonies after two weeks. Then the cells were transfected using an anti-6×His antibody (Clontech, Mountain View, CA). The colonies expressing the highest levels of NYGGF4 were selected for cell proliferation studies.

2.7. Cell growth analysis using NYGGF4 stable transfected 3T3-L1 cells

To analyze the growth of stably transfected 3T3-L1 cells, cells were seeded in 96-well culture plates at a density of 5×102 cells/well and cultured in media supplemented with 10% FBS and G418 100 μg/ml penicillin and 100 μg/ml streptomycin sulfate, in a 5% CO2 incubator at 37 °C. Before transfection, the cells were serum deprived for 48 h to synchronize the cell cycle. Before analysis, cells were serum deprived for 48 h to synchronize the cell cycle. Cells were incubated with DMEM containing 10% (v/v) FBS for various time (0 h, 12 h, 18 h, 24 h) after serum deprivation. Cell cycle distributions were assessed using methods described previously (Olivera et al., 1999). Briefly, cells were fixed in 70% ethanol and cellular DNA was stained with propidium iodide. Total DNA content was analyzed in each cell line by flow cytometry.

2.9. Differentiation of 3T3-L1 Preadipocytes

Induction of adipocytic differentiation of 3T3-L1 cells was essentially performed as described elsewhere (Student et al., 1980). Briefly, 3T3-L1 cells were grown in DMEM supplemented with 10% calf serum until confluency. Two days after complete confluence (day 0), cells were cultured in DMEM supplemented with 10% FBS and 0.5 mM 1-methyl-3-isobutylxanthine (Sigma, St. Louis, MO), 0.25 μM dexamethasone (Sigma, St. Louis, MO) and 100 nM insulin (Sigma, St. Louis, MO) for 48 h. From day 2 to 4, the full medium was supplemented with 100 nM insulin only. The cells were then switched back to DMEM containing only 10% FBS for the remaining days. Cultures were replenished every 2 days.

2.10. Oil red O staining

Cells were stained with oil red O and hematoxylin as described previously (Sahai et al., 1994). Briefly, cells were fixed with 4% formalin in phosphate buffer for 30 min at room temperature. After fixation, cells were stained with 0.6% (w/v) filtered oil red O solution (60% isopropanol, 40% water) for 30 min and then counterstained with hematoxylin for 1 min at room temperature. Cells were then washed with water to remove unbound dye, visualized by light microscopy, and photographed.

2.11. RT-PCR analysis

Total RNA was isolated from 3T3-L1 cells using the TRIzol method (Invitrogen, Carlsbad, CA). One microgram of total RNA was converted to cDNA using 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), and an aliquot (10%) of the resulting cDNA was amplified with the primers listed in Table 1. The number of cycles and reaction temperatures used in the semiquantitative RT-PCR assay were optimized to provide a linear relationship between the amount of input template and the amount of PCR product.

Table 2

<table>
<thead>
<tr>
<th>Clone</th>
<th>Number of non-redundant clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ob-specific</td>
<td>Identified genes 120</td>
</tr>
<tr>
<td></td>
<td>Identified ESTs 80</td>
</tr>
<tr>
<td></td>
<td>Unidentified ESTs 16</td>
</tr>
<tr>
<td></td>
<td>Total 216(550*)</td>
</tr>
<tr>
<td>N-specific</td>
<td>Identified genes 112</td>
</tr>
<tr>
<td></td>
<td>Identified ESTs 78</td>
</tr>
<tr>
<td></td>
<td>Unidentified ESTs 20</td>
</tr>
<tr>
<td></td>
<td>Total 210(450*)</td>
</tr>
</tbody>
</table>

Ob-specific: obese subjects specific; N-specific: normal-weight subjects specific.
* Number of total clones sequenced.
3. Results

3.1. SSH

We used SSH to identify novel genes that are differentially expressed in abdominal subcutaneous fat between obese (BMI > 30 kg/m²) and normal-weight (BMI from 18 kg/m² to 25 kg/m²) subjects. With the statistics analysis, there are no other significant differences between the two groups except the BMI index and weight. None of the subjects were diabetic. After the SSH work, 426 non-redundant clones were identified. Overview of the SSH results are shown in Table 2. The quality control data during the SSH procedure are shown in supplemented data.

3.2. Identification and sequence analysis of NYGGF4 gene

After the SSH, a new clone without homology to known genes was identified and selected for further investigation, which demonstrated increased expression in obese than in normal-weight subjects. The sequence was submitted to the Genbank Database as a novel gene named as NYGGF4 with accession numberAY317148. The nucleic acid sequence and the deduced amino acid sequence are shown in Fig. 1. An open reading frame extends from nucleotide positions 32–784 and encodes a protein of 250 amino acids with an estimated molecular mass of 28.27 kDa. The initiating ATG codon (nucleotides 32–34) is preceded by a TGA stop codon (nucleotides 2–4) triplets upstream and conforms to a consensus initiation sequence (Kozak, 1999). A stop codon is located at nucleotide 750 and a potential polyadenylation signal AATAAA beginning at nucleotide 1262 of the NYGGF4 cDNA.

The amino acid sequence analysis of the predicted NYGGF4 protein using the BLASTp program revealed that the peptide has significant homology to the human BAA91333 unnamed protein (83%, Genbank Accession No. AK00708.1), whose function is unknown. The predicted NYGGF4 protein has neither signal peptide nor transmembrane sequences. Using the Simple Modular Architecture Research Tool (SMART), the predicted NYGGF4 protein has a Phosphotyrosine-Binding (PTB) domain at amino acids 86–232.

3.3. Differential expression of NYGGF4 in human obesity

The differential expression of the new gene was confirmed by RT-PCR experiments. We performed amplification of GAPDH gene as control. In Fig. 2, the new gene was expressed at a higher level among the obese than in normal-weight subjects, which was consistent with the results of SSH.
3.4. Pattern of NYGGF4 mRNA expression in various human tissues

We examined the expression of NYGGF4 mRNA in human adipose tissue by northern blot analysis (Fig. 3A). Using a labeled probe, we observed that NYGGF4 hybridized to one transcript of 1.5 kb. To examine the distribution of NYGGF4 in other human tissues, multiple tissue northern blots were independently hybridized with the same probes. As shown in Fig. 3B, an ~1.5 kb transcript was shown expressed primarily in heart and skeletal muscle, but hardly detectable in other tissues including brain, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung and peripheral blood leukocyte. The size of the transcripts was similar to that found in human adipose tissue.

3.5. Intracellular localization of NYGGF4

Since subcellular localization of NYGGF4 protein within the cells may provide key information regarding the function of the gene, we performed transient transfection of an NYGGF4-GFP expression vector into 293-T and 3T3-L1 cells. The signal was detected by confocal microscopy 24 h after transfection. As shown in Fig. 4, NYGGF4 localized in the cytoplasm of both 293-T and 3T3-L1 cells. But interestingly, the NYGGF4 were localized to a punctuate structure within the cytosolic compartment of 3T3-L1 cells. The punctuate structure may be caused by the new gene distribution to the Golgi, endoplasmic reticulum, endosomes, lysosomes, or mitochondria, which needs investigation in the future (Jobbagy et al., 2000). All these indicated that NYGGF4 might be a cytoplasmic protein and further work is needed for biochemical and functional characterization of the protein.

3.6. Ecotopic expression of NYGGF4 increases 3T3-L1 proliferation

Obesity is the result of an expansion of individual adipocytes and an increase in the number of adipocytes. NYGGF4 is expressed at a higher level in obese than in normal-weight subjects, and the amino acid sequence analysis of NYGGF4 indicated it might function as a signaling molecule and play a role in cell proliferation. So we chose 3T3-L1 cells, which is one of the most studied preadipocyte cell lines in obesity research (Gregoire et al., 1998; MacDougald and Lane, 1995), to characterize the effect of ectopic expression of NYGGF4 on cell growth. 3T3-L1 cells were stably transfected with either an empty expression vector (pcDNA3.1Myc/His B) or an NYGGF4 expression vector. The stably transfected cells were grown under normal growth conditions (DMEM containing 10% FBS and 100 μg/ml G418). Cell growth was analyzed by MTT assays every day for a period of 7 days. The results are presented as means ± SD in triplicate. (Section 3.6).
7 days. Untransfected cells served as controls. As shown in Fig. 5, ectopic expression of NYGGF4 resulted in a much higher proliferation rate compared with controls, while the cells transfected with empty vector and untransfected cells did not show any difference in cell growth rate.

3.7. NYGGF4 drives more 3T3-L1 cells into S-phase

The effect of NYGGF4 on 3T3-L1 cell growth was further examined by cell cycle analysis of stably transfected cells. 3T3-L1 cells transfected with empty vector or untransfected cells served as controls. In Fig. 6, more than 80% cells of all cell lines stayed in G1 phase after starvation of serum for 48 h. The cells were then stimulated to reenter the cycle with serum. The percentage of NYGGF4-expressing 3T3-L1 cells in the S-phase compartment increased 12 h after serum stimulation, reaching a peak at 18 h. Conversely, the control cells were still in G0/G1 phase 12 h after treatment, and the percentage of parental cells in S-phase at 18 and 24 h after treatment was less than the NYGGF4 transfected cells. From these results, we can see that ectopic expression NYGGF4 drives more 3T3-L1 cells into S-phase. These results suggest that NYGGF4 may play a crucial role in preadipocyte proliferation, and may be necessary for obesity progression.

3.8. Analysis of the differentiated phenotype of NYGGF4 transfectants

After confluent, 3T3-L1 preadipocyte become growth arrested, and then terminally differentiate into adipocytes with hormonal induction. Therefore, to investigate whether NYGGF4 affects preadipocyte differentiation, we used the 3T3-L1 cell line stably
expressing \textit{NYGGF4} and assayed its ability to differentiate into adipocytes. We investigated the \textit{NYGGF4} gene effect on adipocytic differentiation of 3T3-L1 from day 0 to day 14. In Fig. 7, we found that induced numerous fat droplets appeared as early as day 2. But the lipid droplet number or size did not show any difference between the \textit{NYGGF4}-expressing 3T3-L1 cells and the controls at day 2, day 6 and day 14 of differentiation. Furthermore, we analyzed the expression of adipocyte-specific molecular markers by RT-PCR assay in these cells during the differentiating treatment. We chose peroxisome proliferator-activated receptor gamma (PPAR-gamma), adipocyte lipid binding protein gene (AP2) and lipoprotein lipase (LPL), whose
expression are up-regulated during 3T3-L1 preadipocyte differentiation (Gregoire et al., 1998). In Fig. 8, cell lines expressed NYGGF4 showed consistent trend on expression of these marker genes and no differences at the expression level comparable with the control cells at day 0, day2, day 4, day6 and day 14.

4. Discussion

Obesity is a multifactorial disease resulting from the interactions between susceptibility genes and environmental factors. The obesity gene map (Perusse et al., 2005) reveals that putative loci affecting obesity-related phenotypes are found on all autosomes and on chromosome Y. The numbers of genes, markers and chromosomal regions that have been thus far associated or linked with obesity phenotypes is increasing and reach now about 200.

In the present study, we described the identification and characterization of a new gene, NYGGF4, which showed a higher expression level among obese than in normal-weight subjects using a suppression subtractive hybridization technique. Northern blot analysis showed a unique pattern of gene expression of NYGGF4 in human various tissues. NYGGF4 mRNA expression was found in adipose tissue, heart and skeletal muscle. The expression pattern of NYGGF4 raises the question about its possible role in these tissues, but at present, detailed are unclear.

The function of the NYGGF4 genes is currently unknown. The presence of multiple sequence motifs or domains found in known proteins may give some hints to understanding the function of the protein. Sequence analysis reveals that the deduced NYGGF4 protein has a PTB domain at amino acids 86–232, which was initially identified based on the ability to recognize phosphorylated tyrosine residues (van der Geer and Pawson, 1995). The PTB domains are found in many cytoplasmic signaling proteins, allowing them to bind specifically to other polypeptides that are tyrosine-phosphorylated in response to growth factor stimulation (Kavanaugh et al., 1995). These observations suggested that NYGGF4 may be a cytoplasm molecule and have functions related to cell growth. With transient transfection of green fluorescent protein (GFP)-tagged NYGGF4 in 293-T and 3T3-L1 cells we confirmed its cytoplasm distribution. Further evidence for a role of NYGGF4 in cell growth is provided by ectopic transfection of NYGGF4 into the preadipocyte 3T3-L1 cells. First, the MTT assay showed that preadipocytes overexpressing NYGGF4 grew faster than control cells preadipocyte proliferation. Second, cell cycle analysis by flow cytometry showed a remarkably increased percentage of S-phase cells in NYGGF4-overexpressing preadipocytes. All these data showed that NYGGF4 increases the preadipocyte 3T3-L1 cells proliferation. Furthermore, we also investigate the effects of NYGGF4 on 3T3-L1 preadipocyte differentiation and found that the new gene does not affect the differentiation of 3T3-L1 cells. As obesity is the result of an expansion of individual adipocytes and an increase in the number of adipocytes, we think that NYGGF4 may stimulate the growth rate of the 3T3-L1 preadipocyte as a signaling molecule and then play a role in adipogenesis.

In summary, we have described the identification and characterization of a new gene NYGGF4, which was expressed at a higher expression level among obese than in normal-weight subjects. However, the precise molecular function of NYGGF4 remains unclear. The generation of knockout or knockdown cells in which NYGGF4 expression is markedly attenuated, is essential for further characterization of the molecular function of NYGGF4. At present, we do not have such cells, since human adipocytes are highly resistant to transfection. We are now attempting to establish an efficient knockdown system in human adipocytes. The in vivo function of NYGGF4 remains unknown, and further studies are required to elucidate the biological and biochemical roles of NYGGF4 in human adipocyte differentiation.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (Grant Number: 30571978), Health Bureau of Jiangsu Province (Grant Number: RC2002061). We gratefully acknowledge the technical assistance of the First Affiliated Hospital of Nanjing Medical University in adipose tissue sample obtention.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2006.05.008.

References


