Effects of NYGGF4 knockdown on insulin sensitivity and mitochondrial function in 3T3-L1 adipocytes

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Abstract *NYGGF4* is a recently discovered gene that is involved in obesity-associated insulin resistance. It has been suggested that mitochondrial dysfunction might be responsible for the development of insulin resistance induced by NYGGF4 overexpression. In the present study, we aimed to define the impact of down-regulating NYGGF4 expression by RNA interference (RNAi) on the insulin sensitivity and mitochondrial function of 3T3-L1 adipocytes. The results revealed that NYGGF4 knockdown enhanced the glucose uptake of adipocytes, which reconfirmed the regulatory function of NYGGF4 in adipocyte insulin sensitivity. However, an unexpected observation was that knockdown of NYGGF4 reduced intracellular ATP concentration and promoted an increase in mitochondrial transmembrane potential ($\Delta\Psi m$) and reactive oxygen species (ROS) level without affecting mitochondrial morphology or mtDNA. Therefore, the role of NYGGF4 in mitochondrial function remains unclear, and further animal studies are needed to explore the biological function of this gene.

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Introduction

Obesity is a significant health problem that has reached epidemic proportions around the world and is associated with several metabolic and cardiovascular complications (Kopelman 2000; Spiegelman and Flier 2001; Visscher and Seidell 2001). One of the important consequences of obesity is the development of insulin resistance (IR), which is considered to be an important link between adiposity and the associated risks of type 2 diabetes and cardiovascular disease (Reaven 1988; Qatanani and Lazar 2007; Ferrannini et al. 2007). Insulin resistance is defined as a condition in which normal insulin concentrations fail to achieve normal glucose metabolism (Kahn 1978). To date, the mechanisms underlying IR have remained unclear, although numerous studies have indicated that free fatty acids, proinflammatory cytokines, adipocytokines, and mitochondrial function are implicated in the pathogenesis of IR (de Jongh et al. 2004; Kremen et al. 2006; Tilg and Moschen 2006; Kim et al. 2008).

In an earlier study (Wang et al. 2006), we isolated and characterized *NYGGF4*, a novel human gene showing increased expression in obese subjects. NYGGF4 is a 250-amino acid cytoplasmic protein, and a recent study using protemic analyses has indicated that NYGGF4 might be a new interactor of low-density lipoprotein receptor-

related protein 1 (LRP1), which is a multifunctional receptor that is involved in diverse metabolic pathways and cellular signal transduction (Caratù et al. 2007; Herz and Strickland 2001). Further studies by our group revealed that overexpression of NYGGF4 results in insulin resistance in adipocytes by reducing insulin-stimulated glucose uptake, and we suggested that the accompanying mitochondrial dysfunction might be the potential mechanism underlying this effect (Zhang et al. 2009; Zhao et al. 2010b). In addition, we also found that the expression of NYGGF4 mRNA in adipocytes is affected by a variety of factors that are related to insulin sensitivity, including free fatty acids (FFAs), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), leptin, and resistin (Zhao et al. 2010a). Taken together, these results provide evidence that NYGGF4 may be involved in the regulation of adipocyte insulin sensitivity. Considering that the heterologous overexpression of proteins frequently induces aberrant organelle import, incorrect protein folding, and/or altered protein-protein interactions (Baneyx and Mujacic 2004), we aimed in the present study to further elucidate the effects of NYGGF4 on insulin sensitivity and mitochondrial function in 3T3-L1 adipocytes through silencing its expression using small interfering RNAs (siRNAs).

Materials and methods

Cell culture and differentiation

3T3-L1 preadipocytes were grown in high glucose concentration Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, and were maintained in a 5% CO_2 humidified atmosphere at 37 °C. The preadipocytes were induced to differentiate into adipocytes using a previously described method (Herz and Strickland 2001).

shRNA preparation and plasmid construction

A 19-nt short hairpin RNA (shRNA) targeting sequence was designed according to the mouse NYGGF4 mRNA sequence in the Genbank database (NM_001003948). Its specificity was confirmed using the BLAST program (http://www.ncbi.nlm.gov/BLAST). The designed shRNA construct contained a unique 19-nt double-stranded

NYGGF4 sequence presented as an inverted complementary repeat, a loop sequence (5′-TTCAAGAGA-3′), the RNA PloIII terminator (5′-TTTTTT-3′), and 5′ single-stranded overhangs for ligation into BamHI- and BbsI-digested pGPU6/GFP/Neo shRNA expression vector (GenePharma, Shanghai, China). The recombinant vector was named pGPU6-NYGGF4-shRNA. The Negative Control vector (pGPU6-NC-shRNA) contained a nonsense shRNA insert in order to control the effects that may be caused by non-RNAi mechanisms and/or by general side effects associated with shRNA delivery in the experiments. The sequences of the two cDNA fragments (sense strands) are as follows: NYGGF4, 5′-TAAGGTGAATAGACACATT-3′; Negative Control, 5′-GTTCTCCGAACGTGTCACG-3′.

Cell transfection

A total of 10^4 3T3-L1 cells were seeded into each well of a 6-well cell culture plate (Costar, Cambridge, MA, USA). When the cells reached 30–50% confluence, they were transfected with appropriate plasmids (pGPU6-NYGGF4-shRNA or pGPU6-NC-shRNA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At 48 h post-transfection, 800 μg/mLG418 (Sigma, St. Louis, MO, USA) was added to select positive cell clones. Stable cell clones were isolated after 2 weeks.

RNA extraction and quantitative real-time PCR

Total RNAs from the 3T3-L1 cells transfected with pGPU6-NYGGF4-shRNA or pGPU6-NC-shRNA were extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed using an AMV Reverse Transcriptase kit (Promega, Madison, WI, USA) using a random hexamer primer. Real-time PCR was performed using an Applied Biosystems 7300 Sequence Detection System (ABI 7300 SDS; Foster City, CA, USA) by following the manufacturer's protocols. The sequences of the primers and TaqMan probes (Shenggong, Shanghai, China) are shown in Table 1.

Glucose uptake

2-Deoxy-D-[^{3}H] glucose (CIC, Beijing, China) uptake was assayed as described previously (Ceddia et al. 2005). The

Table 1 Sequences for primer/probe sets used in real-time PCR

Gene	Forward primer (5′–3′)	Probe	Reverse primer (5′–3′)
CYTB	TTTTATCTGCATCTGAGTTTAATCCTGT	AGCAATCGTTCACCTCCTCTTCCTCCAC	CCACTTCATCTTACCATTTATTATCGC
28 s	GGCGGCCAAGCGTTCATAG	TGGTAGCTTCGCCCCATTGGCTCCT	AGGCGTTCAGTCATAATCCCACAG
NYGGF4	TTCCTCTCCTATTCCAACACTTC	AGATCTCCACGCCTATTTTCACCA	GGAGCAACCAGTCAGTCACC
β-actin	CCTGAGGCTCTTTTCCAGCC	TCCTTCTTGGGTATGGAATCCTGTGGC	TAGAGGTCTTTACGGATGTCAACGT

stably transfected cells were cultured in 6-well plates and induced to form mature adipocytes. On day 10 of differentiation, the cells were serum starved in DMEM containing 0.5% FBS for 3 h. The cells were then washed twice with phosphate-buffered saline (PBS) and incubated in KRP-HEPES buffer [30 mmol/L HEPES (pH 7.4), 10 mmol/L $NaHCO_3$, 120 mmol/L NaCl, 4 mmol/L KH_2PO_3, 1 mmol/L $MgSO_4$, and 1 mmol/L $CaCl_2$] in the presence or absence of 30 μmol/L LY294002 for 30 min, and then the cells were treated with vehicle or 100 nM insulin for 30 min. Glucose uptake was measured by adding 2 μCi of 2-deoxy-D-[^{3}H] glucose for 10 min. The assay was terminated with two quick washes of ice-cold PBS supplemented with 10 mmol/L D-glucose. The cells were solubilized by adding 200 μL 1 mol/L NaOH to each well. Aliquots of the resultant cell lysate were transferred to scintillation vials for radioactivity counting, whereas the remainder was used for the protein assay.

Electron microscopy

On the 10th day of differentiation, the mature adipocytes were collected after trypsin digestion, washed in fresh PBS (pH 7.4), and fixed in 2.5% glutaraldehyde/4% paraformaldehyde in the same buffer. The cells were then washed in 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide/1.5% potassium ferrocyanide for 1 h, washed in water, stained with 1% aqueous uranyl acetate for 30 min, and then dehydrated through a graded series of ethanol to 100%. The samples were then infiltrated and embedded in TAAB Epon (Marivac Canada Inc., St. Laurent, Canada). Ultrathin sections (60 nm) were cut on a Reichert Ultracut-S microtome, placed onto copper grids, stained with uranyl acetate and lead citrate, and examined on a transmission electron microscope (JEM-1010; JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

Real-time PCR for mitochondrial DNA (mtDNA)

Relative amounts of mtDNA were determined by real-time PCR as previously described (Kaaman et al. 2007). Briefly, DNA was isolated from adipocytes using a DNA extraction kit (Baitaike, Beijing, China) and quantified by spectrophotometry at 260 nm. Two primer sets were used for PCR analysis (the sequences are shown in Table 1). A 110-nt mtDNA fragment within the CYTB gene was used for the quantification of mtDNA. The PCR product has previously been cloned into the plasmid pMD-T 18 and verified by DNA sequencing. Plasmid standards of known copy number were used to generate a log-linear standard curve, from which the CYTB copy numbers of the studied samples could be determined by real-time PCR. A 291-bp region of the nuclear gene for 28S rRNA was used to normalize the results. A standard curve of plasmids containing the 28S fragment was used to determine the copy numbers of

the studied samples. The ratio of mtDNA to nuclear DNA reflects the concentration of mitochondria per cell.

ATP production

On the 10th day of differentiation, the ATP content of the adipocytes was measured using a luciferase-based luminescence assay kit (Biyuntian, Nantong, China). Briefly, the adipocytes were homogenized in an ice-cold ATP-releasing buffer. Light emission was recorded for 30 s using a photon-counting luminometer. The relative ATP level was normalized by protein concentration determined by the BCA method.

Mitochondrial membrane potential (ΔΨm)

The mitochondrial membrane potential was determined using a JC-1 probe as described previously (Woollacott and Simpson 2001). Assays were initialized by incubating adipocytes with 2 μmol/L JC-1 (Beyotime, Nantong, China) for 30 min and the fluorescence of individual cells was detected using a fluorescence-assisted cell sorter (FACS). Mitochondrial membrane potential was determined from the ratio of red fluorescence (excitation, 550 nm; emission, 600 nm) to green fluorescence (excitation, 485 nm; emission, 535 nm).

Reactive oxygen species (ROS) assay

Intracellular ROS generation was determined using 6-carboxy-2, 7-dichlorodihydrofluorescein diacetate (H2-DCFDA) as previously described (Sundaresan et al. 1995). In brief, adipocytes were washed and incubated with H2-DCFDA (Sigma, St. Louis, MO, USA) for 20 min. Cells were then washed several times and harvested in PBS. The fluorescence of H2-DCFDA was detected with FACS (excitation, 488 nm; emission, 530 nm), and the images were captured under a fluorescence microscope.
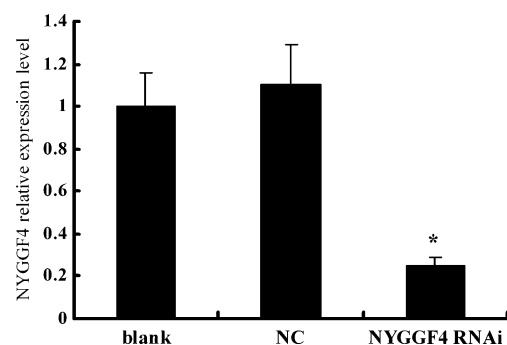

Fig. 1 Knockdown of NYGGF4 mRNA expression by siRNA. NYGGF4 mRNA relative to β-actin in the pGPU6-NYGGF4-shRNA-transfected 3T3-L1 preadipocytes was examined by real-time PCR in comparison with pGPU6-Negative Control (NC)-shRNA-transfected and blank 3T3-L1 preadipocytes. The mRNA inhibitory efficiency was 75% ($P<0.01$ vs. NC adipocytes)
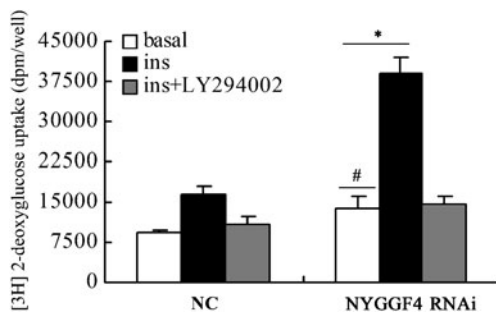
Fig. 2 Effects of NYGGF4 knockdown on adipocyte glucose uptake. NYGGF4 knockdown 3T3-L1 predipocytes and Negative Control (NC) cells were induced to differentiate. On the 10th day of differentiation, the mature adipocytes were pretreated with or without LY294002 for 30 min before being treated with 100 nM insulin. Glucose uptake was then measured for 10 min, as described in the Materials and Methods. Values shown are the means ± SE of three independent experiments performed in triplicate. $^{\#}P<0.05$ vs. basal glucose uptake in NC adipocytes, $^{*}P<0.01$ vs. insulin-stimulated glucose uptake in NC adipocytes.

Statistical analysis

All data are expressed as the means ± SEM. Statistical analysis was performed using one-way ANOVA or Student's t-test with the SPSS 10.0 statistical software package (SPSS Inc., Chicago, IL, USA). The threshold of significance was defined as $P<0.05$.

Results

Suppression of NYGGF4 expression by small interfering RNA

The knock down efficiency of NYGGF4-specific shRNA in 3T3-L1 cells was evaluated by real-time PCR. The relative NYGGF4 mRNA level in transfectants was normalized against the mRNA levels of an endogenous control gene, β-actin. The

value of NYGGF4 mRNA in the cells transfected with Lipofectamine 2000 alone, which was normalized to β-actin, was set as one. As shown in Fig. 1, cells transfected with pGPU6-NYGGF4-shRNA showed a significantly reduced transcription of NYGGF4 mRNA when compared with non-transfected and Negative Control vector pGPU6-NC-shRNA-transfected cells ($P<0.05$). There was no significant difference in mRNA transcription between pGPU6-NC-shRNA-transfected and non-transfected cells ($P>0.05$). The steady state NYGGF4 mRNA level of the pGPU6-NYGGF4-shRNA-transfected cells was 25% that of the Negative Control cells.

Inhibition of NYGGF4 expression increases glucose uptake

To test whether NYGGF4 affects insulin sensitivity, we measured glucose uptake in 3T3-L1 adipocytes with or without NYGGF4 depletion. Basal and insulin-stimulated glucose uptake was significantly upregulated in NYGGF4 knockdown adipocytes compared with negative control cells. Next, we measured insulin-induced glucose uptake enhancement after LY294002 (a PI3-kinase inhibitor) pretreatment. LY294002 was found to completely block insulin-stimulated glucose uptake (Fig. 2) in both cell types, and this result suggested that knockdown of NYGGF4 triggers glucose uptake in adipocytes via a PI3-kinase-dependent signaling pathway.

Effects of NYGGF4 knockdown on mitochondrial morphology and mtDNA number copy

As shown in Fig. 3, the morphology of mitochondria in NYGGF4 knockdown adipocytes was similar to that of the negative control cells. The size and ultrastructure of mitochondria are not altered after NYGGF4 knockdown. The mtDNA copy number per mitochondrion is generally considered to be constant in all mammalian cell types (Robin and Wong 1988); therefore, the mtDNA copy number is
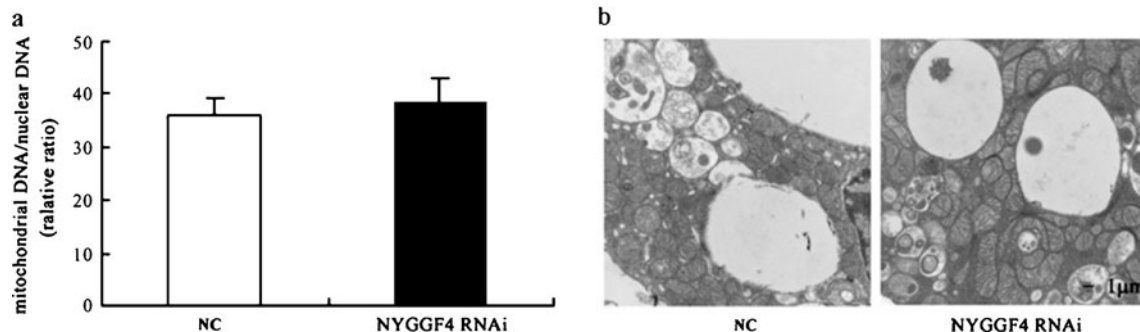

Fig. 3 Effects of NYGGF4 knockdown on the mitochondrial DNA (mtDNA) copy number and mitochondrial morphology in 3T3-L1 adipocytes. NYGGF4 knockdown 3T3-L1 predipocytes and Negative Control (NC) cells were induced to differentiate. On the 10th day of differentiation, cellular mtDNA content was assessed by real-time PCR analysis with primers designed to target the CYTB and 28 S

rRNA genes ($n=6$). The CYTB to 28S rRNA gene ratio reflects the concentration of mitochondria per cell (a). The ultrastructure of mitochondria in the adipocytes was visualized by transmission electron microscopy. The scale bar in the bottom right corner of b represents 1 μm (b)

generally considered as an indicator of cellular mitochondrial number. Using real-time PCR, we assessed the effects of NYGGF4 knockdown on mtDNA copy number, and the results showed that there was no significant difference in mtDNA copy number between the two groups ($P>0.05$).

Effect of NYGGF4 silencing on cellular ATP production

In eukaryotic cells, the mitochondrion is the major platform for energy transduction, producing ATP via oxidative metabolism of nutrients. The efficiency of ATP production reflects the integrity of mitochondrial function. In the present study, we found that total cellular ATP production was dramatically decreased in NYGGF4-silenced adipocytes compared with the negative control cells ($P<0.01$, Fig. 4). These results indicated that the suppression of NYGGF4 expression affected mitochondrial function.

Effects of NYGGF4 knockdown on $\Delta\Psi m$ and intracellular ROS levels

As shown in Fig. 5, the ratio of red to green fluorescence increased from 31% in the control to 44% in NYGGF4 knockdown adipocytes, indicating that $\Delta\Psi m$ was elevated when the expression of NYGGF4 was suppressed. Mitochondria are the primary source of endogenous ROS, which are generated as a by-product of metabolic reactions within this organelle (Andreyev et al. 2005). As shown in Fig. 5, ROS levels in NYGGF4 knockdown adipocytes were strikingly enhanced, as indicated by the greater fluorescence in the presence of the compound DFCDA.

Discussion

RNA interference (RNAi) is a conserved biological mechanism triggered by double-stranded RNA from exogenous (small interfering RNA, siRNA) or endoge-
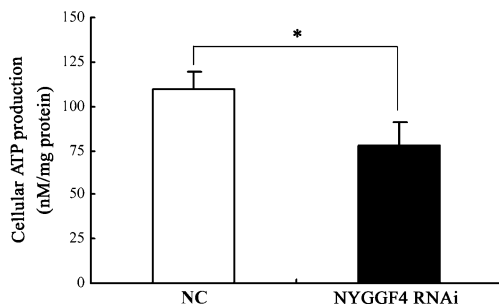
Fig. 4 Effects of NYGGF4 silencing on cellular ATP production. NYGGF4 knockdown 3T3-L1 predipocytes and Negative Control (NC) cells were induced to differentiate. On the 10th day of differentiation, cellular ATP production was measured and normalized to protein concentrations ($n=6$). *$P<0.01$ vs. the NC adipocytes
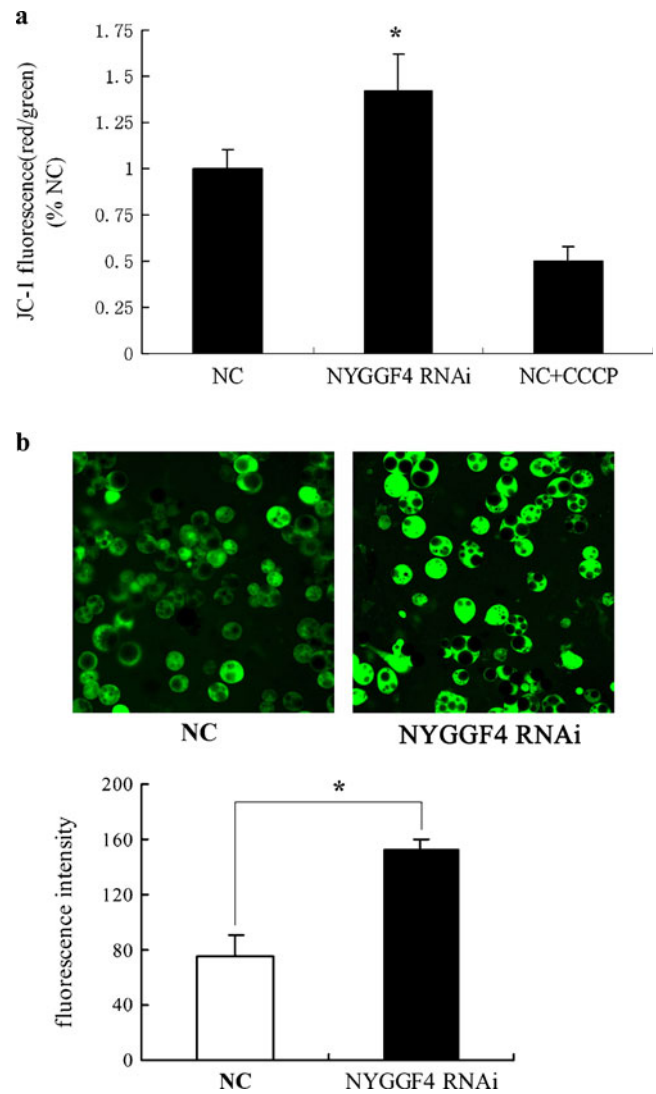
Fig. 5 Effects of NYGGF4 knockdown on $\Delta\Psi m$ and intracellular ROS levels. NYGGF4 knockdown 3T3-L1 predipocytes and Negative Control (NC) cells were induced to differentiate. On the 10th day of differentiation, mitochondrial membrane potential ($\Delta\Psi m$) was analyzed by calculating the ratio of cells with red and green fluorescence generated by JC-1. Fluorescence of the harvested adipocytes was detected by FACS. CCCP, which uncouples the membrane potential, serves as a positive control. *$P<0.01$ vs. the NC adipocytes (**a**). ROS levels were determined by measuring the fluorescence of DCFDA using a confocal laser scanning microscope and FACS. *$P<0.01$ vs. the NC adipocytes (**b**) ($n=6$)

nous (microRNAs, miRNA) origin that inhibits gene expression at the transcriptional level (Shrey et al. 2009). In recent years, this ancient cellular antiviral mechanism has been widely used as a gene silencing tool for investigating gene function with high specificity and efficiency (Zhai et al. 2009). In the current study, we successfully constructed an shRNA expression vector to knock down endogenous NYGGF4 expression. The data showed that transfection with NYGGF4-shRNA specifically targeted endogenous NYGGF4 mRNA levels. These

effects were not observed in the cells transfected with the negative control shRNA when compared with normal 3T3-L1 adipocytes.

Our results revealed that inhibition of the expression of NYGGF4 increased the insulin-stimulated glucose uptake of 3T3-L1 adipocytes. In addition, glucose uptake in response to insulin could be completely abrogated by pretreatment with the PI3-kinase inhibitor LY294002 in both NYGGF4 knockdown adipocytes and negative control cells. These results suggested that silencing of NYGGF4 enhances glucose uptake in adipocytes via a PI3-kinase-dependent signaling pathway. This finding is consistent with our previous results, which showed that overexpression of NYGGF4 inhibits insulin-stimulated glucose uptake through an attenuated PI3K signaling pathway (Zhang et al. 2009). It is worth pointing out that the basal glucose uptake was also up-regulated in NYGGF4 knockdown adipocytes. However, the molecular mechanism underlying this effect remains unknown.

There is growing evidence that mitochondrial dysfunction plays an important role in the pathogenesis of insulin resistance (Kim et al. 2008). Our previous study also showed that overexpression of NYGGF4 in 3T3-L1 adipocytes decreased mitochondrial mass, mitochondrial DNA, ATP synthesis, and elevated intracellular ROS level, which indicated that mitochondrial dysfunction might be responsible for the development of NYGGF4-induced insulin resistance. Therefore, in this study, we further investigated the effect of NYGGF4 knockdown on mitochondrial function in adipocytes. The results showed that suppression of NYGGF4 had no obvious effect on the morphology and number of mitochondria. Furthermore, decreased ATP synthesis, elevated $\Delta\Psi m$, and increased intracellular ROS production were observed in NYGGF4 knockdown adipocytes. These observations suggested that suppression of NYGGF4 affected mitochondrial function to some extent, which appears to contradict previous results. We considered the following as possible reasons for the observed phenomena. Firstly, NYGGF4 is a novel inter-actor of LRP1 and inhibition of its expression would alter the function of LRP1 in lipoprotein and lipid metabolism, which might result in decreased ATP synthesis. The elevated glucose uptake and $\Delta\Psi m$ in NYGGF4 knockdown adipocytes would be expected to generate more ATP in order to compensate for the lost ATP production, and a sustained elevation of $\Delta\Psi m$ is known to elevate the production of ROS. Secondly, many studies have demonstrated that overexpression of a recombinant protein sometimes increases the likelihood of misfolding through the routine use of strong promoters and high inducer concentrations. The misfolded proteins are retained in the endoplasmic reticulum (ER) and their accumulation may interfere with ER function that can cause ER stress (Ami et al. 2009; Le Bras et al. 2006).

Sustained ER stress would result in the release of ER calcium at the mitochondria-associated membrane, thereby triggering loss of mitochondrial membrane potential and promoting apoptosis (Ron and Walter 2007; Scorrano et al. 2003; Deniaud et al. 2008). Hence, we speculate that mitochondrial dysfunction induced by NYGGF4 overexpression is possibly due to the misfolding of the NYGGF4 protein that results in ER stress and mitochondrial apoptosis. However, the exact effect of NYGGF4 on mitochondrial function remains to be determined.

In summary, the present study showed that inhibition of NYGGF4 expression enhanced glucose uptake in 3T3-L1 adipocytes, but decreased ATP synthesis and elevated the levels of $\Delta\Psi m$ and ROS. Additional studies using animal models will be essential for an enhanced understanding of the biological function of NYGGF4.

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