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CLINICAL STUDY

LYRM1, a novel gene promotes proliferation and inhibits apoptosis of preadipocytes

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Abstract

Objective: To characterize a novel gene, *Homo sapiens* LYR motif containing 1 (*LYRM1*), that is highly expressed in omental adipose tissue of obese subjects.

Methods and results: RT-PCR and western blot analysis confirmed that both mRNA and protein levels of *LYRM1* were higher in omental adipose tissue of obese subjects than in normal weight subjects. RT-PCR analysis demonstrated that *LYRM1* expression is widely distributed, with the highest levels of expression occurring in adipose tissue. A fusion protein of LYRM1 and green fluorescent protein as well as western blot analysis were used to identify the subcellular localization of LYRM1 in the nucleus. Based on Oil red O staining and the expression profile of specific differentiation markers, ectopic LYRM1 expression was not found to significantly affect adipogenesis. MTT assays and cell cycle analysis showed that LYRM1 promotes preadipocyte proliferation, and data from annexin V-FITC and caspase-3 activity assays further determined that LYRM1 can inhibit apoptosis of preadipocytes.

Conclusions: By increasing cell proliferation and lowering the rate of apoptosis, LYRM1 has the potential to modulate the size of the preadipocyte pool and influence adipose tissue homeostasis.

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Introduction

Obesity results from interactions between genetic, environmental, and psychosocial factors, is an important public health problem in the developed world and a growing problem in the developing world. Currently, over 600 genes, markers and chromosomal regions have been identified as associated with or linked to human obesity phenotypes (1). However, in $>95\%$ of severe obesity cases the responsible genes remain unknown (2). Thus, identification and characterization of novel genes and proteins associated with obesity remain an important issue.

In a previous report, we analyzed the gene expression profiles of omental adipose tissue of obese individuals (age 45.2 ± 14.5 years; body mass index (BMI) 31.6 ± 0.4; insulin sensitivity index 0.016 ± 0.004) and normal control individuals (age 47.8 ± 16.7 years; BMI 20.7 ± 1.2; insulin sensitivity index 0.017 ± 0.007) to identify novel genes that may be implicated in the genetic susceptibility or the pathological consequences of obesity. We identified 216 genes up-regulated in obese subjects and 210 genes up-regulated in normal subjects (3). By using suppression subtractive hybridization, a cluster of clones identical to one gene commonly comprised one to three clones. Notably, a novel gene termed *Homo sapiens* LYR motif containing 1 (*LYRM1*) in GenBank (GenBank accession number NM_020424), encoding a wholly novel protein (GenBank accession number NP_065157) was identified by eight clones, suggesting an important role in human obesity. Therefore, this gene was selected for further analysis. The *LYRM1* derives from an mRNA of 1589 bp with a 369 bp open reading frame (ORF), which encodes a 122-amino acid protein (Fig. 1A). NCBI Map Viewer analysis revealed that the *LYRM1* gene is located on chromosome 16 and is composed of four exons. A sequence comparison of mouse and rat *LYRM1* orthologous genes suggests that *LYRM1* represents a novel gene highly conserved across primates and rodents (Fig. 1B). In this study, we report the tissue distribution and subcellular localization of *LYRM1*. Furthermore, we examined the effect of LYRM1 on cell differentiation, proliferation, and apoptosis *in vitro* by establishing a stable preadipocyte cell line overexpressing LYRM1, and found that while overexpression of LYRM1 in preadipocytes does not affect the differentiation of preadipocytes, it stimulates

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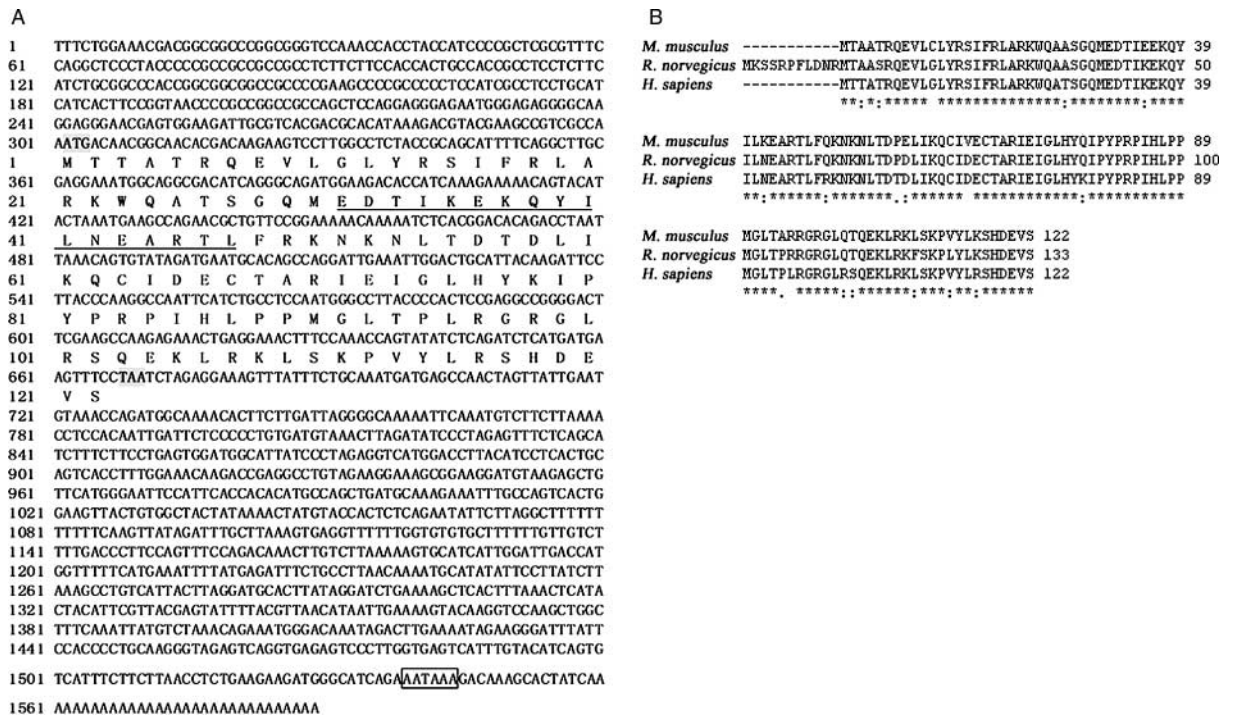

Figure 1 Nucleotide and deduced amino acid sequence of *LYRM1*. (A) The nucleotide and amino acid positions are provided at the beginning of each line of sequence. The ATG translation start codon and TAA stop codon are shaded in gray and the first polyadenylation signal is boxed. The 17-amino acid peptide synthesized for immunization is underlined. (B) Sequence comparison of the deduced amino acid sequence of mouse, rat, and human LYRM1 protein. Asterisks mark amino acids conserved among the three sequences compared.

preadipocyte proliferation and protects preadipocytes from apoptosis.

Materials and methods

Samples

All human omental adipose tissues were obtained as previously indicated (3). Six obese subjects and six normal subjects were randomly selected to form two groups.

For determination of *LYRM1* tissue distribution, RT-PCR was carried out using as the templates eight normalized, first strand cDNA preparations from polyA$^+$ RNA extracted from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas multiple tissue cDNA (MTC) Panel I, Clontech). The adipose tissue cDNA sample was prepared from the normal group. The liver tissue cDNA sample was prepared from a cadaver donor (age 52; BMI 22.35).

RT-PCR

According to the manufacturer's instructions, total RNA was extracted from tissue with TRIZOL reagents (Stratagene, San Diego, CA, USA). Samples of 200 ng total RNA were subjected to RT using random primers with Moloney murine leukemia virus reverse transcriptase (Promega). An aliquot (10%) of the resulting cDNA was amplified with primers listed in Table 1. The number of cycles and reaction temperatures were optimized to provide a linear relationship between the amount of input template and the amount of PCR product.

Preparation of anti-LYRM1 polyclonal antibody

A synthetic 17 residue oligopeptide (EDTIKEKQYIL-NEARTL) corresponding to the LYRM1 protein was conjugated to keyhole limpet hemocyanin (KLH) with *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (Wako, Osaka, Japan) and used as an antigen. Antisera were raised in New Zealand rabbits by s.c. injections of KLH-conjugated oligopeptide. Antiserum was purified using an oligopeptide-conjugated column and elution with 0.2 M glycine buffer (pH 2.5). Collected antibody was dialyzed against PBS, concentrated using a Centricon YM-10 cartridge (Millipore, Bedford, MA, USA), and stored at −20 °C.

Western blotting

Whole adipose tissue lysates were prepared by homogenization in modified RIPA buffer (50 mM Tris–HCl (pH 7.4), 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS) and M-PERTM Mammalian Protein Extraction

Table 1 Primer sequences used.

Gene name	Product size (bp)	Reverse and forward primer (5′–3′)	Temperature (°C)	Cycles
LYRM1	453	R: GGCTTGCGAGGAAATGGCAGGCGAC F: CACAGGGGGAGAATCAATTGTGGAG	64	26
GAPDH	983	R: TGAAGGTCGGAGTCAACGGATTTGGT F: CATGTGGGCCATGAGGTCCACCAC	68	22
AP2	582	R: GAAGCTTGTCTCCAGTGAA F: CCAAGCTTTTGTCAACTGT	56	32
C/EBPα	391	R: CAGTTTGGCAAGAATCAGAGCA F: GGGTGAGTTCATGGAGAATGG	58	30
FAS	499	R: AGCCACGAGTGAGTGTACGGGAG F: GGACAGGACAAGACAAAAAGGG	56	30
PPARγ	266	R: GACCACTCGCATTCCTTT F: CCACAGACTCGGCACTCA	58	30
β-actin	240	R: CTGTGCTATCCCTGTACG F: TGCCAATGGTGATGACCT	58	28

Reagent (Pierce Chemical Company, Rockford, IL, USA). Protease Inhibitor Cocktail (Pierce Chemical Company) was added during homogenization and tissue debris was removed by centrifugation. Cytoplasmic and nuclear extracts from adipose tissue were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Chemical Company). Protein concentration was determined using a BCA Protein Assay Reagent Kit (Pierce Chemical Company) and western blots were performed as described previously (3).

Subcellular localization of LYRM1 protein based on GFP fusion product

A green fluorescent protein (GFP)-LYRM1 fusion protein was expressed from the plasmid, pEGFP-N2-LYRM1. The ORF (nucleotides 401–769) of *LYRM1* was amplified by PCR, inserted into the BamH I/Xho I sites of the pEGFP-N2 vector, and confirmed by DNA sequencing. For transfection, cells were plated on coverslips placed in six-well plates and cultured for 24 h. Transfections were performed according to the manufacturer's protocol (Invitrogen). As a control, pEGFP-N2 vector without an LYRM1 insert was transfected into cells under the same conditions. GFP fluorescence was examined using a confocal laser microscope (Zeiss, Gottingen, Germany) 24 h post-transfection.

Cell culture and differentiation of 3T3-L1 preadipocytes

3T3-L1, COS-7, Hela, and NIH3T3 cells (ATCC, Manassas, VA, USA) were maintained in DMEM with 10% FCS (Biomedia, Boussens, France), 100 units/ml penicillin, and 50 μg/ml streptomycin (Life Technologies Inc.) at 37 °C in 5% CO_2. To induce differentiation, 2-day post-confluent 3T3-L1 preadipocytes (day 0) were exposed to a differentiation cocktail (100 μM methylisobutylxanthine, 0.25 μM dexamethasone, 1 μg/ml insulin). Two days later (day 2), cells were switched to a medium containing 1 μg/ml insulin to incubate for another 2 days (day 4). The cells were then returned to DMEM/10% FCS until day 8 with fresh medium provided every 2 days.

Establishment of a stable cell line overexpressing LYRM1

For overexpression studies, pcDNA3.1Myc/HisB-LYRM1 was constructed by inserting the PCR amplified ORF (nucleotides 401–769) of *LYRM1* into BamH I/Xho I sites of the vector. The sequence of the inserted ORF (368 bp) was confirmed by DNA sequencing. Expression vectors carrying the LYRM1 coding sequence, or empty vectors, were transfected into 3T3-L1 cells and 48 h-later neomycin (G418, Roche) selection (800 μg/ml) was started. After 2 weeks, individual colonies were isolated, propagated, and the expression of the LYRM1-6×His fusion protein was confirmed by western blot. The anti-6×His antibody used was from Clontech. Colonies expressing the highest levels of LYRM1 were selected for studies.

Oil red O staining

For Oil red O staining, adipocytes were washed thrice with PBS and fixed with 4% formalin in phosphate buffer for 30 min at room temperature. After fixation, cells were washed twice with PBS and stained with 0.6% (w/v) filtered Oil red O solution (60% isopropanol, 40% water) for 60 min at room temperature. Cells were washed with water to remove unbound dye, visualized by light microscopy, and photographed.

MTT assay

Adipocytes (2×10^2/well) were seeded in 96-well plates and maintained in serum-free DMEM for 24 h until they were adherent. Media were then changed to DMEM supplemented with 10% FBS. Cell growth was

monitored for seven consecutive days using the Cell Proliferation MTT Kit (Roche Diagnostics) as recommended by the manufacturer. Absorbance values at 560 and 660 nm were recorded by an ELISA reader and the difference between these values was recorded as the optical density.

Cell cycle assay

Cells (2×10^6/750 mm^2) were cultured in DMEM with 10% FBS. Once adhered, the cells were washed with PBS and starved in serum-free DMEM for 24 h to synchronize. Cell cycle analysis was initiated at various timepoints (0, 6, 12, 18, and 24 h) after replacement of the starvation medium with complete medium. Cultured cells were harvested using trypsin/EDTA and washed twice with PBS. Aliquots of 2×10^6 cells were centrifuged, fixed in 70% ethanol, and stained with 500 µl propidium iodide (PI) solution (100 µg/ml RNase and 50 µg/ml PI in 1×PBS). Labeled cells were analyzed using a BD FACScan and data were analyzed using CellQuest software.

Apoptosis assay

Cells were cultured in FBS-free DMEM or TNF-α (6 nM) for 24 h to induce apoptosis. Cells were then harvested using trypsin/EDTA, washed with PBS, resuspended in 1 ml binding buffer, and stained with 10 µl annexin V-FITC and 10 µl PI at room temperature for 5 min (Biovision, CA, USA). The fluorescence of FITC and PI was analyzed using flow cytometry.

Measurement of caspase-3 activity

After inducing apoptosis, cells were collected and washed with PBS. Activity of caspase-3 was assayed using a commercially available kit (Sigma) according to the manufacturer's protocol. Additional assays were performed using inhibitors included in the kit.

Statistical analysis

Each experiment was performed at least thrice. All values are presented as the mean±S.D. Statistical analyses were performed using Student's *t*-test and statistical significance was when $P<0.05$.

Results

Differential expression of LYRM1 *in human obesity*

Differential expression of *LYRM1* was confirmed by RT-PCR (Fig. 2A) and western blot analysis (Fig. 2B). As shown in Fig. 2, *LYRM1* was expressed at a higher level in samples from obese versus normal weight subjects at both the mRNA ($^*P<0.001$) and protein ($^{**}P<0.05$) levels.

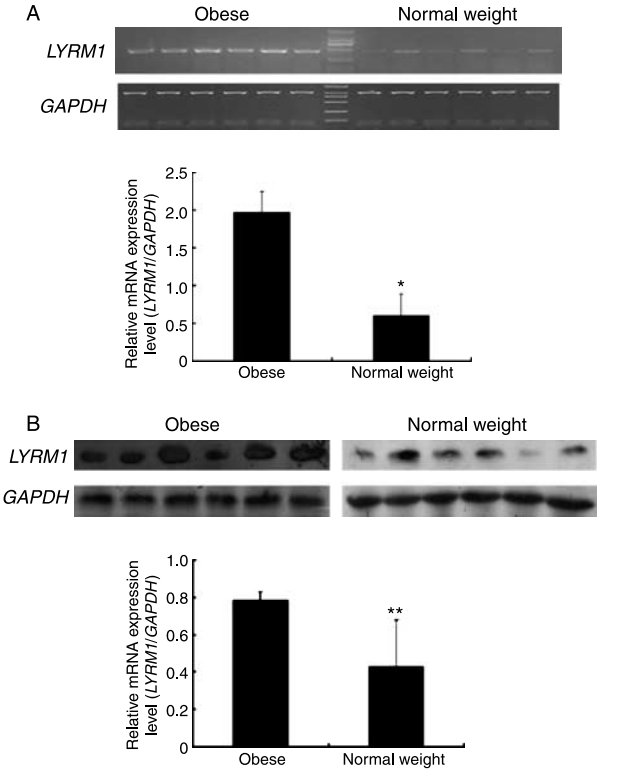

Figure 2 Differential expression of *LYRM1* in human obesity. The differential expression of *LYRM1* was confirmed by RT-PCR (A) and western blot (B) analysis. Results of RT-PCR and western blot analysis are expressed as the ratio between the intensity of bands corresponding to *LYRM1* versus the intensity of bands corresponding to *GAPDH*. The expression level of *LYRM1* in obese subjects was found to be significantly higher than in normal weight subjects at both the mRNA ($^*P<0.001$) and protein ($^{**}P<0.05$) levels.

Tissue distribution of LYRM1 *mRNA expression*

To examine the tissue distribution of *LYRM1* mRNA, we performed RT-PCR analysis of various human tissues (Fig. 3). Among the human MTC panel I including eight human adult tissues, liver showed the highest level of *LYRM1* expression. We further compared the expression of *LYRM1* between adipose tissue and liver tissue and found the level of *LYRM1* in adipose tissue to be higher ($P<0.001$).

Subcellular localization of LYRM1 protein

We examined the subcellular localization of LYRM1 by transient transfection of 3T3-L1, COS-7, Hela, and NIH3T3 cells with a fusion protein of LYRM1 and GFP. Fluorescent imaging demonstrated that the fusion

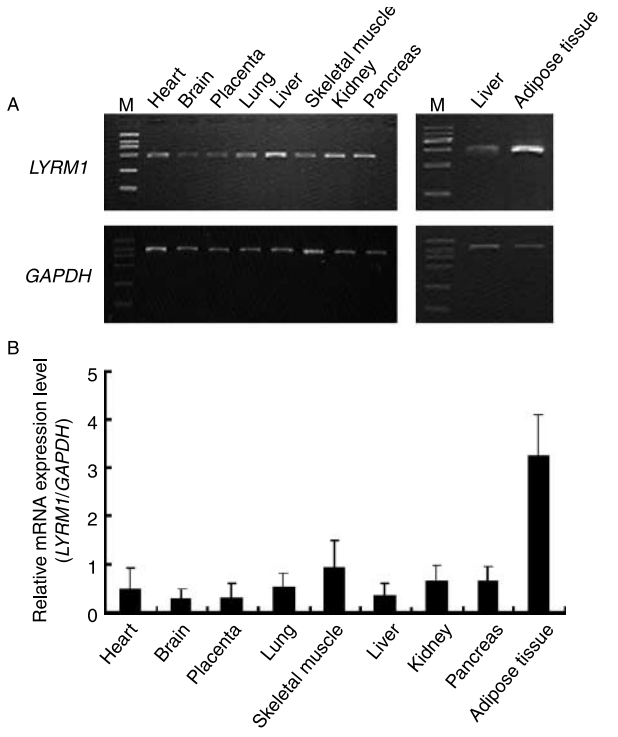

Figure 3 Tissue distribution of *LYRM1* mRNA expression. (A) *LYRM1* mRNA levels were analyzed in a panel of human tissues using RT-PCR. (B) Results are expressed as the ratio between the intensity of bands corresponding to *LYRM1* versus the intensity of bands corresponding to *GAPDH*. Among the human tissues tested, adipose tissue showed the highest levels of the *LYRM1* expression.

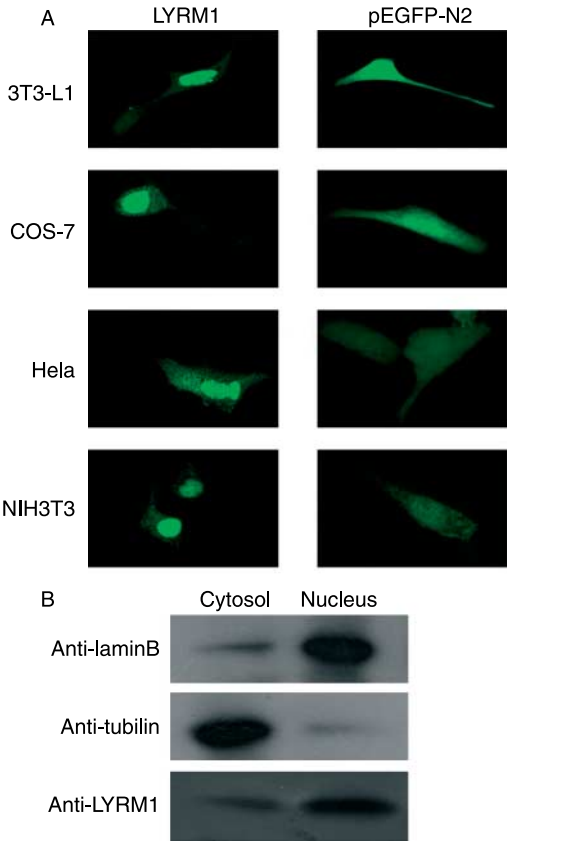

Figure 4 Subcellular localization of LYRM1. (A) Cells were transiently transfected with LYRM1-pEGFP-N2 and observed by live-cell confocal microscopy. Fusion proteins were observed mainly in the nucleus of cells. (B) Cytoplasmic and nuclear extracts were obtained from adipose tissue for western blot analysis and LYRM1 was detected primarily in the nuclear fractions, with very little LYRM1 protein detected in the cytoplasm.

protein localized primarily to the nucleus, whereas GFP alone distributed throughout the whole cell without any specific compartmentalization (Fig. 4A). For further confirmation of the nuclear localization of LYRM1, cytoplasmic and nuclear extracts were obtained from adipose tissue for western blot analysis. Anti-LYRM1 antibody binding was visualized in the nuclear fractions from adipose tissue, with very little detection of LYRM1 in the cytoplasm (Fig. 4B).

Effect of LYRM1 on cell differentiation

To investigate whether LYRM1 affects preadipocyte differentiation, we used a 3T3-L1 cell line stably overexpressing LYRM1 and assayed its ability to differentiate into adipocytes. As a control, 3T3-L1 cells were transfected with pcDNA 3.1/His. In Fig. 5A, we showed that neither the number of lipid droplets nor their size differed between the LYRM1-overexpressing 3T3-L1 cells and the control cells until day 8 following stimulation of differentiation. Furthermore, we analyzed the expression of adipocyte-specific molecular markers by RT-PCR at various timepoints during stimulation of differentiation. The adipocyte-specific markers monitored included adipocyte lipid-binding protein gene (*AP2*), CCAAT enhancer-binding protein α (*CEBPA*), fatty acid synthetase (*FASN*), and peroxisome proliferators-activated receptor γ (*PPAR*γ), whose expression are up-regulated during 3T3-L1 preadipocyte differentiation. In Fig. 5B, data from RT-PCR assays showed that expression levels of these marker genes detected in cell lines overexpressing *LYRM1* on days 0, 2, 4, 6, and 8 were similar to the control cells at the same timepoints ($P > 0.05$).

Effect of LYRM1 on cell proliferation

MTT assays were used to evaluate the proliferation of preadipocytes overexpressing LYRM1. An increased growth rate of LYRM1-overexpressing cells was found compared with control cells ($^{*}P = 0.001$, $^{**}P < 0.001$). LYRM1 also had an effect on the cell cycle. Flow cytometry analysis of cell cycle distribution (Fig. 6B) detected a significantly higher percentage of 3T3-L1 cells overexpressing LYRM1 were in the S phase of the cell cycle compared with control cells ($^{**}P < 0.001$).

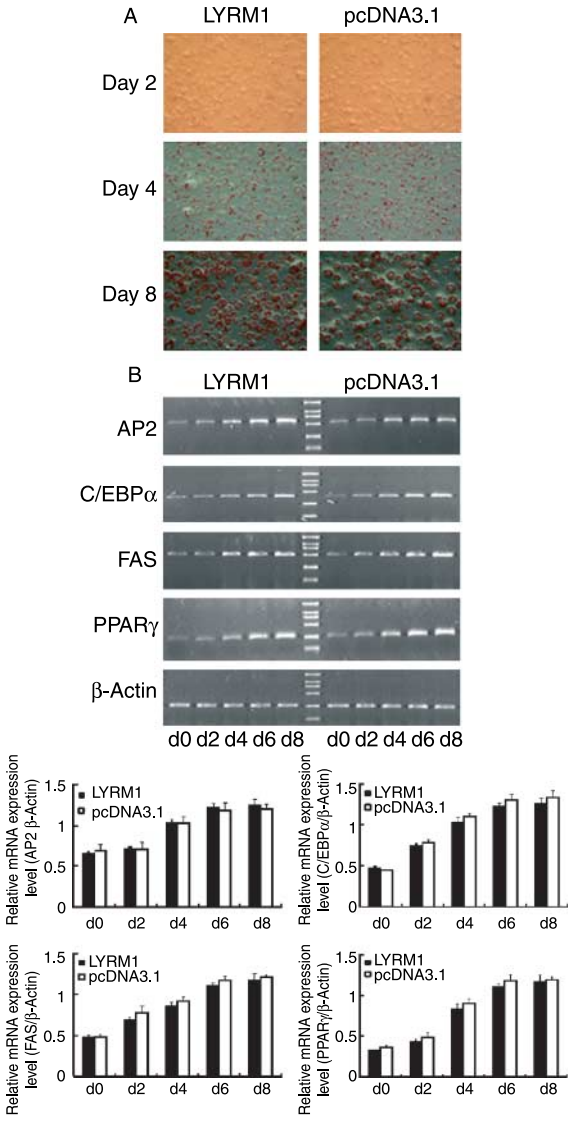

Figure 5 Effect of LYRM1 on cell differentiation. (A) 3T3-L1 cells were transfected with pcDNA3.1Myc/HisB-LYRM1 or empty vector and stimulated to differentiate over 8 days. On day 2, 4, and 8, cells were fixed and stained with Oil red O to visualize lipids present. (B) Expression of adipocyte-specific molecular markers was analyzed by RT-PCR at various timepoints during the stimulation of differentiation (day 0, 2, 4, 6, and 8). No difference in expression levels of these markers was found between cells with or without exogenous expression of LYRM1 ($P>0.05$).

Effect of LYRM1 on cell apoptosis

It has shown that apoptosis can be induced in 3T3-L1 cells by serum deprivation and addition of TNF-α (4, 5). To study the effect of LYRM1 on cell apoptosis, 3T3-L1 cells were cultured in FBS-free DMEM with or without TNF-α (6 nM) for 24 h. Binding studies with annexin V-FITC (Fig. 7A) showed that LYRM1 protects 3T3-L1 preadipocytes from serum deprivation-induced apoptosis ($^{**}P<0.001$) and TNF-α-induced apoptosis ($^{**}P<0.001$) respectively. Caspase-3 activity was also assayed. Data in Fig. 7B showed that LYRM1 inhibits serum deprivation-induced apoptosis ($^{**}P<0.001$) and TNF-α-induced apoptosis ($^{**}P<0.001$) respectively.

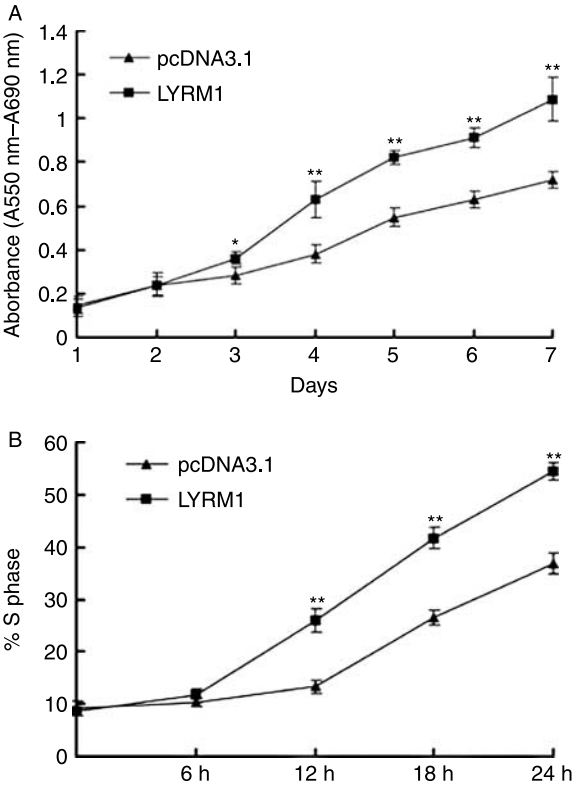

Figure 6 Effect of LYRM1 on cell proliferation. The effects of LYRM1 on cell proliferation were detected by MTT assays (A) and cell cycle analysis (B). Data from both assays showed that LYRM1 promotes the proliferation of preadipocytes ($^{*}P=0.001$, $^{**}P<0.001$).

Discussion

Obesity is a chronic and costly condition whose incidence is rapidly increasing throughout the world. Obesity is considered a major risk factor for type 2 diabetes (6) and has also been linked to cancer and immune dysfunction (7). Understanding the genes involved in the development of obesity is important for the development of new therapies that directly target molecular mechanisms underlying obesity. In the present work, we reported *LYRM1* that expression is up-regulated in obese subjects as a novel gene that might play a role in obesity.

Multi-tissue expression analysis of *LYRM1* was performed since a multi-tissue expression analysis may provide additional information regarding the function of a novel gene. The expression of *LYRM1* was found in all nine tissues tested, with the highest levels of expression in adipose tissue. These results support a role for *LYRM1* in human obesity.

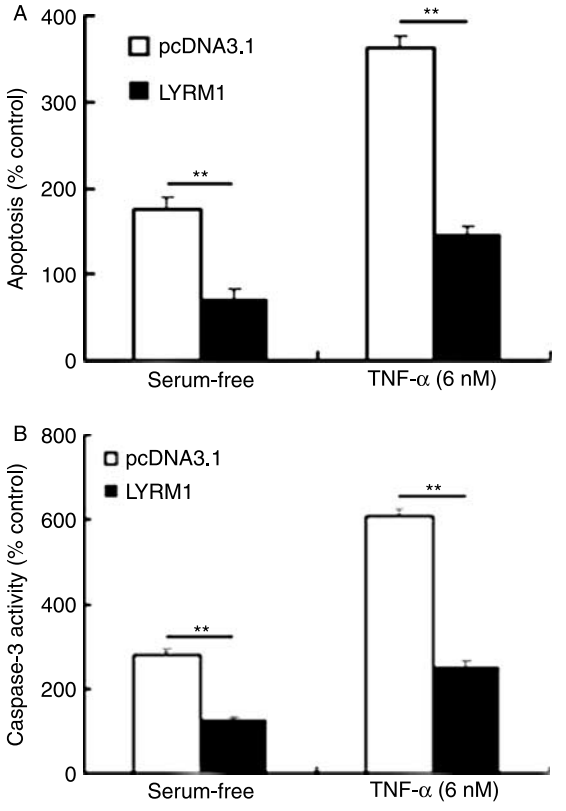

Figure 7 Effect of LYRM1 on cell apoptosis. Apoptosis was assayed by binding of annexin V-FITC (A) and detection of caspase-3 activity (B). LYRM1 was found to inhibit the apoptosis of preadipocytes (**$P<0.001$) in both studies.

We used subcellular localization studies to determine the localization of LYRM1 in various cell lines and human adipose tissue because the subcellular localization may provide key information regarding the function of a gene. Expression of GFP-tagged LYRM1 and western blot analysis showed that LYRM1 localized primarily to the nucleus. Further studies are needed to identify the role of nuclear localization in the function of LYRM1.

Adipose tissue, as a metabolic and endocrine organ, plays an essential role in the regulation of energy balance (8). Accordingly, adipocytes are emerging as a potential therapeutic target for obesity, type 2 diabetes, and cardiovascular disease (9). Adipose tissue mass reflects the number and average volume of adipocytes, in particular, the balance between cell acquisition and cell loss (10–12). Therefore, the proliferation of adipocyte precursors and their differentiation into mature adipocytes, combined with the apoptosis of preadipocytes, both contribute to the development of obesity in mammals (13–17). The 3T3-L1 murine preadipocyte line is one of the established models of adipogenesis and is believed to accurately reflect the *in vivo* biological features of adipocytes (7, 18). Although 3T3-L1 cells are mouse cells, the basic mechanisms for fat development appear to be similar in both rodent and human cells (19). Therefore, considerable progress has been made in understanding the molecular mechanisms of adipocyte biology using the 3T3-L1 preadipocyte cell line (20) as a model. Accordingly, we chose this system in which to investigate the effects of LYRM1 on preadipocyte differentiation, proliferation, and apoptosis. By establishing a stably transfected 3T3-L1 cell line overexpressing LYRM1, we found that: i) LYRM1 does not affect the differentiation of 3T3-L1 cells, as shown by Oil Red staining and the expression of adipocyte-specific molecular markers; ii) the result of the MTT assay indicated that LYRM1 causes the promotion of cell population growth of 3T3-L1 preadipocytes and cell cycle analysis showed a dramatic increase in the percentage of cells in S phase; and iii) LYRM1 can prevent apoptosis induced by serum deprivation and addition of TNF-α in preadipocytes with the analysis of annexin V-FITC and caspase-3 activity. In summary, our data demonstrate that by increasing cell proliferation and lowering apoptotic rate, LYRM1 has the potential to affect the size of the preadipocyte pool and influence adipose tissue homeostasis.

In conclusion, we characterized several original features of the novel gene *LYRM1*, including its up-regulation in obese subjects, its highest expression level in adipose tissue, its nuclear distribution, and its effect on the number of preadipocyte cells present. However, the precise functional properties of this gene remain to be clarified and await further investigation. For example, the need to evaluate the same differentiation, proliferation, and apoptotic parameters in other cell lines overexpressing LYRM1 versus knockdown or knockout cells with attenuated LYRM1 expression. Further studies are also required to elucidate the *in vivo* functions of LYRM1, which would provide valuable insight into its role in obesity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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