Inhibition of preadipocyte differentiation and adipogenesis by zinc-α2-glycoprotein treatment in 3T3-L1 cells

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ABSTRACT

Aims/Introduction: Zinc-α2-glycoprotein (ZAG) is associated with the loss of adipose tissue in cancer cachexia, and has recently been proposed to be a candidate factor in the regulation of bodyweight. The aim of the study was to investigate the effects of ZAG on the proliferation and differentiation of 3T3-L1 preadipocytes.

Materials and Methods: 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) spectrophotometry, Oil Red O staining, intracellular triglyceride assays, real-time quantitative reverse transcription polymerase chain reaction and transient transfection methods were used to explore the action of ZAG.

Results: Ectopic ZAG expression significantly stimulates 3T3-L1 cells proliferation in a dose- and time-dependent manner. The maximum influence of ZAG on proliferation was 1.43-fold higher than what was observed in control cells. This effect was observed 144 h after transfection with 0.16 µg of murine ZAG (mZAG) plasmid ($P < 0.001$). The intracellular lipids content in mZAG over-expressing cells were decreased as much as 37% when compared with the control cells after differentiation ($P < 0.05$, $P < 0.01$). The messenger ribonucleic acid levels of peroxisome proliferators-activated receptor-γ (PPARγ), CCAAT enhancer-binding protein-α (C/EBPα) and the critical lipogenic gene, fatty acid synthase (FAS), are also downregulated by up to 50% in fully differentiated ZAG-treated adipocytes. ZAG suppresses FAS messenger ribonucleic acid expression by reducing FAS promoter activity.

Conclusions: Zinc-α2-glycoprotein stimulates the proliferation and inhibits the differentiation of 3T3-L1 murine preadipocytes. The inhibitory action of ZAG on cell differentiation might be a result of the attenuation of the expression of PPARγ, C/EBPα and the lipogenic-specific enzyme FAS by reducing FAS promoter activity. (J Diabetes Invest, doi: 10.1111/jdi.12046, 2013)

KEY WORDS: 3T3-L1 preadipocytes, Differentiation, Zinc-α2-glycoprotein

INTRODUCTION

Adipose tissue consists of mature adipocytes, preadipocytes, macrophages, fibroblasts and adipose-derived stem cells. Preadipocytes are capable of propagating and differentiating into mature adipocytes. The number of mature adipocytes determines the number of fat cells that exist in the organism throughout its lifespan[1]. Meanwhile, the size of fat cells depends on lipid accumulation in the adipocytes. The preadipose 3T3-L1 cell line is a convenient cell culture model for the investigation of both the adipogenesis program and the identification of the factors that regulate the physiology of mature adipocytes.

Zinc-α2-glycoprotein (ZAG or AZGP1) is a secreted 43 kDa glycoprotein that was first isolated from human plasma, and was subsequently identified in the liver, breast, lung and prostate tissue[2]. Recent work from both Liverpool and our group has shown that ZAG is also expressed in mouse, rat, and human adipose tissue and adipocytes[3–6]. ZAG levels were initially reported to be correlated with bodyweight loss in cancer patients with cachexia[7]. Furthermore, a growing body of evidence shows that ZAG levels in the serum, and adipose tissue of obese patients and obese mice are significantly lower relative to subjects and mice of normal weights[5,8,9]. ZAG levels are negatively correlated with bodyweight and body fat mass[5,10,11]. The administration of ZAG in mice dramatically diminishes bodyweight and fat mass of normal, ob/ob and high-fat-diet- (HFD) induced obese mice[5,12,13]. In addition, our recent study has shown that the rs4215 (A/G) single nucleotide polymorphism in the ZAG gene is associated with obesity in the north Chinese Han population[14]. The expression of the lipogenetic enzymes, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), is significantly downregulated in ZAG-treated mice,

whereas the lipolytic enzyme hormone-sensitive lipase (HSL) is dramatically upregulated in both adipose and liver tissue of these mice. These results suggest that ZAG reduces body fat by regulating lipid metabolism[5,15]. In light of the beneficial effects of ZAG in decreasing body fat, this protein has been suggested to be a therapeutic target of anti-obesity therapeutics[2,12,13]. However, the detailed regulatory mechanisms of ZAG in the context of lipogenesis remain unclear.

This is the first study to investigate the effects of ZAG on the proliferation and differentiation of 3T3-L1 preadipocytes, and the possible mechanism by which this glycoprotein exerts its effects.

MATERIALS AND METHODS

Plasmid Construction

The generation of the pcDNA3.1(−)-murine ZAG (mZAG) expression plasmid that contains full-length mZAG cDNA coding sequences (+1 ± 924 bp) was described in our previous studies. Further *in vitro* and *in vivo* experiments confirmed that the ZAG protein could be successfully expressed in both 3T3-L1 preadipocytes and mice[5,6]. The pGL$_3$-hFAS (−622 ± 3 bp)-Luc (hFAS 625-Luc) vector, which contains the human FAS (hFAS) gene 5′-promoter fragment (spanning −622 to +3 bp) was constructed as described previously[16]. Briefly, human genomic deoxyribonucleic acid (DNA) was extracted from the blood leukocytes of a healthy individual using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The hFAS gene promoter was amplified using the forward primer 5′-TATTAACCACCCGTGTGCGCATTGGGCCG-3′ and the reverse primer 5′-CTCTAGGCCGGCGCCGACGCTATTTAAAC-3′ (GeneBank NC_000017.10). The hFAS625-Luc plasmid was generated by ligating the polymerase chain reaction (PCR) products and the pGL$_3$-Basic vector (Promega Corp., Madison, WI, USA), both of which were digested with *Sal*I and *Hin*dIII restriction enzymes. DNA sequencing was used to confirm the plasmid's sequence. The pcDNA3.1(–) vector was used to balance the total amount of transfected plasmid in the transfection assays, and pRL-SV40 (Promega Corp.) was used as an internal control plasmid.

Cell Culture and Differentiation of 3T3-L1 Preadipocytes

Murine preadipocyte 3T3-L1 cells were purchased from the cell center of the Chinese Academy of Medical Sciences in China. The cells were cultured using Dulbecco's modifiedf Eagle medium (DMEM)-F12 that was supplemented with 10% bovine calf serum (BCS), 100 U/mL penicillin and 100 U/mL streptomycin. The cells were maintained in a 5% CO$_2$ 95% air atmosphere in a 37°C incubator, as described previously[17]. The cells were routinely passaged at below 70% confluence.

3T3-L1 cell differentiation was carried out as previously described[17]. Briefly, differentiation was initiated 2 days after 100% confluence with 10% FBS DMEM-F12 medium that contained 3 μg/mL insulin, 10 μmol/L dexamethasone and 0.5 mmol/L 3-isobutyl-1-methylxanthine (designated as day 0).

After 3 days, the medium was replaced with 10% fetal bovine serum DMEM-F12 that contained 3 μg/mL insulin (day 3) for a further 2 days. The medium was then changed to DMEM-F12 that was supplemented with 10% FBS (day 5) and refreshed every 2 days. Over 90% of the cells were observed under an inverted phase contrast Olympus microscope (Olympus, Tokyo, Japan) to accumulate lipid droplets in the cytoplasm.

### Transient Transfection and 3-(4,5-Dimethylthiazol-2-yl) 2,5-Diphenyl Tetrazolium Bromide Assay

3T3-L1 preadipocytes (1 × 10^4/well) were seeded and maintained overnight in 10% BCS DMEM-F12 medium. Lipofectamine 2000 (Invitrogen Life Technologies, Gran Island, NY, USA) was used to carry out the pcDNA3.1(–)-mZAG expression plasmid transfection experiment. The DNA concentration in the transfection reagent was 0.5 μg/μL. The cell growth was monitored at 24, 36, 48, 72, 96, 120 and 144 h after the transfection using the 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) method, as described previously. For the 96, 120 and l44 h experimental time-points, a second transfection was carried out 72 h after the first transfection[18,19]. Briefly, the cells were washed, 50 μL MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide; 1 mg/mL) was added, and the cells were incubated at 37°C for 4 h. A cell lysis solution (100 μL/well, including 10% w/v sodium dodecyl sulfate [SDS], 5% isobutanol and 0.01 N HCL) was added, and the cells were incubated overnight. The optical density (OD) at 620 nm was recorded using an enzyme-linked immunosorbent assay (ELISA) reader (Anthos Labtec, Wals, Austria).

At the same time, 3T3-L1 cells (2 × 10^4/well) were seeded in 24-well plates. After transfection with 0.2 μg/well mZAG expression plasmid, the cells were lysated, then western blot was carried out to observe whether or not ZAG overexpresses in 3T3-L1 cells. As shown in Figure 1, ZAG expression in ZAG administrated cells significantly increased in comparison with the control, as we described previously[6].

Transient Transfection, Oil Red O Staining and Measurement of Intracellular Triglyceride Content

The preadipocytes were seeded at a cell density of 2 × 10^4 per well, and the differentiation was initiated according to the aforementioned protocol. mZAG expression plasmids (0.2, 0.4, 0.8 μg/well; $n = 3$) were transfected into the adipocytes on days 8, 10, 12 and 14 after differentiation. A total of 48 h later, Oil Red O staining was carried out as described previously[17,18,20]. Briefly, the cells were washed gently and fixed with 4% fresh formaldehyde (Sigma, St. Louis, MO, USA) for 1 h at room temperature. The cells were then stained with 0.6% (w/v) filtered Oil Red O solution (Ameresco, Solon, OH, USA) for 2 h. The pictures were taken by an inverted phase contrast Olympus microscope (Olympus). The Oil Red O dye that was retained in the cells was eluted with 600 μL/well isopropanol, and was quantified by measuring the OD at 492 nm using an ELISA reader.
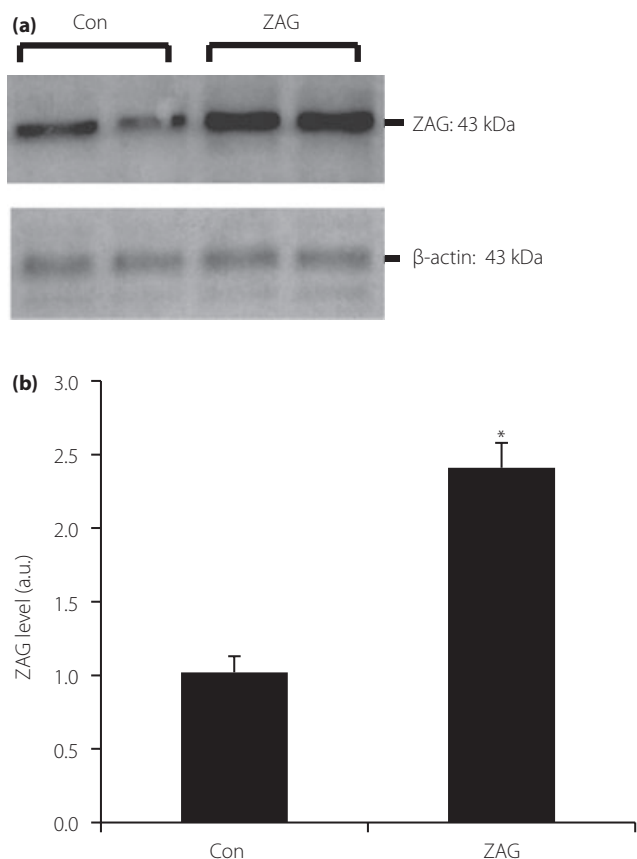
Figure 1 | Zinc-α2-glycoprotein (ZAG) expression in 3T3-L1 cells transfected with or without the ZAG expression plasmid. (a) ZAG level was determined by western blot in 3T3-L1 cells treated with 0.2 μg/well murine ZAG (mZAG) expression plasmid for 48 h. (b) Densitometric analysis of the blot in panel A. *$P < 0.001$ vs control in cell lysis. a.u., Arbitrary unit.

The cellular triglyceride contents were measured using a commercial Triglyceride Assay Kit (Zen bio Inc., Research Triangle Park, NC, USA) according to the manufacturer's instructions.

Real-Time Fluorescence Quantitative Reverse Transcription Polymerase Chain Reaction Analysis

The cell total RNA was extracted using an EZNA Total RNA kit (OmegaBio-Tek, Doraville, GA, USA). The complementary deoxyribonucleic acid (cDNA) synthesis was carried out using the SuperScript First-Strand Synthesis System kit (Invitrogen). The quantitative reverse transcription polymerase chain reaction (RT–qPCR) was carried out in 96-well plates with SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and an ABI 7500 PCR detection system (Applied Biosystems, Foster City, CA, USA). 18S rRNA was used as a reference housekeeping gene for normalization, and the results are expressed as fold change of the cycle threshold (C_t) value relative to the control sample using the $2^{-\Delta\Delta C_t}$ formula[21]. The primer details are available in Table S1.

Dual Luciferase Reporter Gene Assay System

On days 4 and 7 after cell differentiation, mZAG plasmid (0.125, 0.25 or 0.5 μg/well; $n = 3$) was transfected into the adipocytes in combination with the luciferase reporter gene plasmid hFAS625-Luc (0.5 μg/well) and the internal control plasmid pRL-SV40 (16.5 ng/well) using Lipofectamine 2000. These cells were lysed 48 h later, and 20 μL/well of the lysate was used to measure both firefly and renilla luciferase activities using a commercially available Dual-Luciferase® Reporter Assay System kit (Promega) in an automated optical immunoassay analyzer (Beijing Pilot Biotechnology Co., Fengtai district, China). The firefly luciferase activities were adjusted using the renilla luciferase activities.

Statistical Analyses

All of the experiments were carried out in triplicate. At least three samples were used for each experiment. The data are given as the mean ± standard error of the mean. The mean value of the control group in all of the experiments was defined as 100%, and the relative value of the experimental groups was obtained by comparison with the control group. The statistical analyses were carried out between the control groups and the experimental groups by analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant.

RESULTS

ZAG Promotes the Proliferation of 3T3-L1 Preadipocytes

First, ZAG can overexpress in ZAG expression plasmid-transfected 3T3-L1 preadipocytes as shown in Figure 1 and our previous study[6]. We next investigated the effect of ZAG on the proliferation of 3T3-L1 preadipocytes by transfecting the ZAG expression plasmid into 3T3-L1 cells. The results of the MTT assays showed that ZAG gradually and significantly promoted 3T3-L1 cell growth in a dose-dependent manner (from 0.02 to 0.16 μg/well). This effect increased with longer culture periods the extension of action time (48–144 h) when compared with the control cells (Figure 2, △$P < 0.05$, *$P < 0.01$, **$P < 0.001$). The maximal stimulatory action was noted to be 1.43-fold above that which was observed in the control cells. This maximum effect was observed for the 0.16 μg/well ZAG treatment at 144 h (Figure 2, 143.4 ± 4.0% vs 99.8 ± 2.6%, **$P < 0.001$).

ZAG Inhibits Intracellular Lipid Accumulation in Adipocytes

To investigate the effect of ZAG on intracellular lipid accumulation in adipocytes, 3T3-L1 preadipocytes were induced to differentiate using a standard differentiation cocktail medium. As shown in Figure 3b, on day 6 after differentiation, over 90% of the cells were observed to accumulate lipid droplets in the cytoplasm under an inverted phase contrast Olympus microscope. With the extension of differentiation, lipid droplets in cytoplasm of adipocytes becomes larger and the size of adipocytes increases as shown by Figure 3c,e. After transfecting the ZAG expression plasmid into 3T3-L1
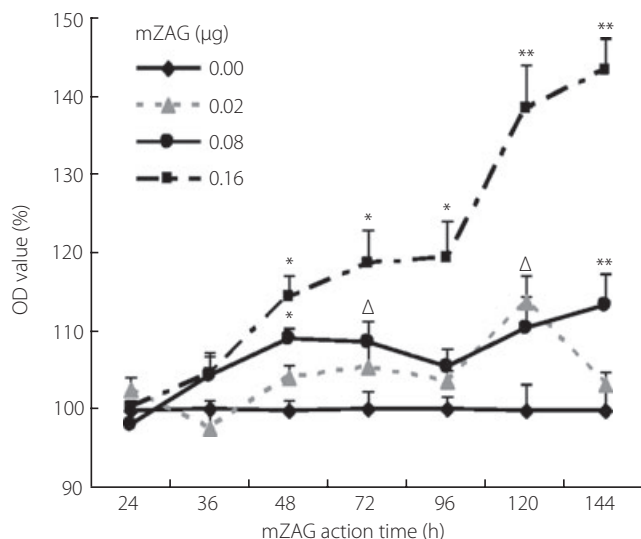
Figure 2 | The effect of zinc-α2-glycoprotein (ZAG) on the 3T3-L1 preadipocyte proliferation. 3T3-L1 cells were plated in 96-well plates at a density of 1×10^4 cells/well. After 24 h, these cells were transfected with the murine ZAG (mZAG) expression plasmid (0.02, 0.08 or 0.16 μg/well; $n = 9$). The cell proliferation was monitored using the MTT method at 24, 36, 48, 72, 96, 120 and 144 h after transfection. For the 96, 120 and 144 h time-points, a second transfection was carried out 72 h after the first transfection. The data represent mean ± standard error of the mean in three independent experiments. $^{\triangle}P < 0.05$, $^*P < 0.01$, $^{**}P < 0.001$ compared with the control group (0 μg).

cells, lipid droplets in cytoplasm became smaller and the size of adipocytes was shrunk without the number changes of adipocytes as shown by Figure 3d, f. In order to further quantify the intracellular lipid accumulation, the methods of Oil Red O staining and direct TG measurements were used. As shown by Figure 4a, the OD value of the Oil Red O-eluted solution significantly decreased in the ZAG-treated fully differentiated adipocytes in comparison with the control cells ($^{\triangle}P < 0.05$, $^*P < 0.01$). The maximal inhibitory effect was observed on day 14 after differentiation at a ZAG plasmid concentration of 0.4 μg/well. For this treatment, the OD value decreased by 25% compared with the control cells (Figure 4a, $75.2 \pm 1.1\%$ vs $99.9 \pm 2.7\%$, $P < 0.01$). Based on direct TG measurements, ZAG transfection reduced the intracellular TG content of differentiated adipocytes. The maximal suppression effect occurred on days 14 after differentiation at a ZAG plasmid concentration of 0.4 μg/well. In these conditions, the OD was reduced by 37% in comparison with the control cells (Figure 4b, $62.8 \pm 1.6\%$ vs $99.8 \pm 5.0\%$, $P < 0.01$). We observed a slight trend showing that ZAG expression enhanced the inhibition of lipid accumulation with longer differentiation protocols. This effect was observed using both Oil Red O staining and direct TG measurements.

ZAG Decreases the Expression of Peroxisome Proliferator-Activated Receptor-γ, CCAAT/Enhancer Binding Protein-α and FAS in Differentiating and Fully Differentiated Adipocytes

To investigate whether the reduced lipid accumulation in ZAG overexpressed cells was a result of the inhibition of adipocyte differentiation, we examined the expression of adipogenic markers in these cells. The expression of peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein-α (C/EBPα), master regulators of adipogenesis, was markedly suppressed in ZAG-transfected adipocytes during differentiation process at concentrations of 0.8 and 1.6 μg/well ZAG ($^{\triangle}P < 0.05$, $^*P < 0.01$, $^{**}P < 0.001$) as shown in Figure 5a. The maximal suppression effect of ZAG on the expression of PPARγ and C/EBPα was 42 and 35%, respectively, in comparison with the control cells. These data show that ZAG inhibits 3T3-L1 preadipocyte differentiation by lowering the expression of PPARγ and C/EBPα.

As PPARγ and C/EBPα are also key regulators of lipogenesis, and play a vital role in the maintenance of the adipocyte phenotype, we next investigated the expression of these proteins in fully differentiated adipocytes. Mature 3T3-L1 adipocytes were transfected with ZAG for 48 h, and the expression level of PPARγ and C/EBPα was determined by RT–qPCR. As shown in Figure 5b, overexpression of ZAG downregulated PPARγ and C/EBPα mRNA levels in fully differentiated adipocytes in a dose-dependent manner. The expression of PPARγ and C/EBPα were suppressed by 50 and 49%, respectively, at the concentrations of 1.6 μg/well ZAG (Figure 5b, $49.8 \pm 3.6\%$ vs $100.9 \pm 1.7\%$, $50.7 \pm 5.1\%$ vs $100.5 \pm 3.3\%$, $^{**}P < 0.001$).

As FAS is essential enzymes for fatty acid synthesis and plays an important role in lipid metabolism, the expression of FAS in differentiating and fully differentiated adipocytes was also evaluated. In accordance with the results obtained in the experiment of PPARγ and C/EBPα, ZAG also efficiently suppressed FAS mRNA levels both in differentiating and fully differentiated adipocytes by 42 and 45%, respectively, at the concentrations of 1.6 μg/well ZAG (Figure 5b; $57.7 \pm 2.1\%$ vs $101.2 \pm 3.9\%$, $55.3 \pm 4.1\%$ vs $100.5 \pm 3.8\%$, $^{**}P < 0.001$). Taken together, these results show that ZAG impairs the ability of adipocytes to synthesize fat by inhibiting PPARγ, C/EBPα and FAS expression.

ZAG Suppresses FAS Promoter Activity in Differentiating and Fully Differentiated Adipocytes

The mechanisms by which ZAG inhibited FAS expression were examined by cotransfecting the luciferase reporter gene plasmid hFAS625-Luc and the ZAG expression plasmid into differentiating and fully differentiated adipocytes. Figure 6 show that the overexpression of ZAG at 0.25 and 0.5 μg/well in differentiating adipocytes decreased significantly the luciferase activities by 18 and 30%, respectively, when compared with the control cells ($^{\triangle}P < 0.05$, $^{**}P < 0.001$). Consistent with this result, overexpression ZAG in fully differentiated adipocytes also markedly suppressed the luciferase activity. This inhibitory action was
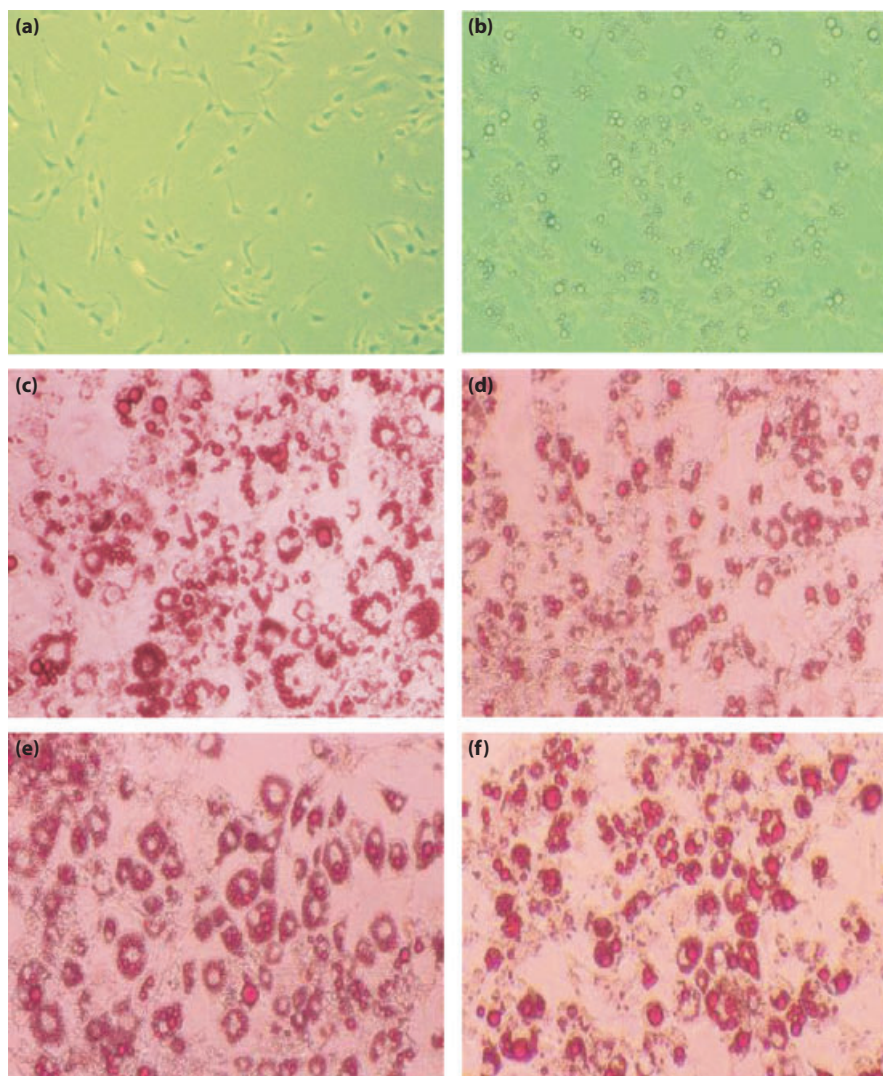
Figure 3 | 3T3-L1 preadipocytes differentiation and effect of zinc-α2-glycoprotein (ZAG) on the lipid accumulation of adipocytes as determined by the Oil Red O staining photographically. (a) 3T3-L1 preadipocyte. (b) Adipocytes on day 6 after induction of differentiation. Adipocytes on day 8 after induction of differentiation transfected with (c) control or (d) ZAG expression plasmid (0.4 μg/well, $n = 3$). Adipocytes on day 12 after induction of differentiation transfected with (e) control or (f) ZAG expression plasmid (0.4 μg/well, $n = 3$). All experiment was carried out on three replicates for each treatment (magnification:× 100).

dose-dependent. The maximal effect was observed at 0.5 μg/well ZAG where the luciferase activity was inhibited by 47% (Figure 4, 52.8 ± 2.3% vs 100.0 ± 6.8%, **$P < 0.001$).

DISSCUSSION
Adipocytes play a vital role in lipid homeostasis and energy balance by regulating TG storage and free fatty acid release. Furthermore, these cells secrete various bioactive molecules that are referred to as adipokines[1]. ZAG is an adipocyte-secreted adipokine, and was recently proposed to be a candidate gene for bodyweight regulation. In the present study, we showed that ZAG was able to promote 3T3-L1 preadipocyte proliferation in a dose- and time-dependent manner, and inhibited the differentiation of 3T3-L1 preadipocytes, which showed significantly reduced intracellular lipid accumulations.
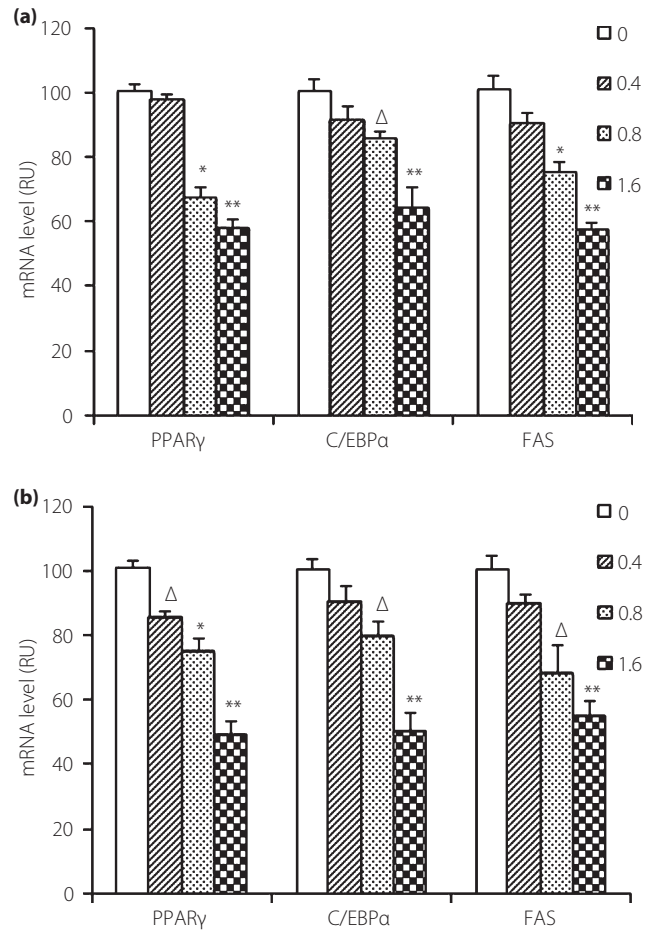
Zinc-α2-glycoprotein was initially identified in 1998 as the lipid-mobilizing factor that was associated with the loss of adipose tissue in cancer patients with cachexia, a severe life-threatening wasting syndrome[22]. Certain studies have investigated the effect of ZAG on the tumor cell proliferation, including oral tumors[23] and uterine carcinoma cells[24]. The results of these studies suggested that ZAG could inhibit tumor cell proliferation. However, in the present study, we showed that ZAG stimulates preadipocyte proliferation in a dose- and time-dependent manner, which is a beneficial phenomenon in the context of obesity given that promoting preadipocyte proliferation often
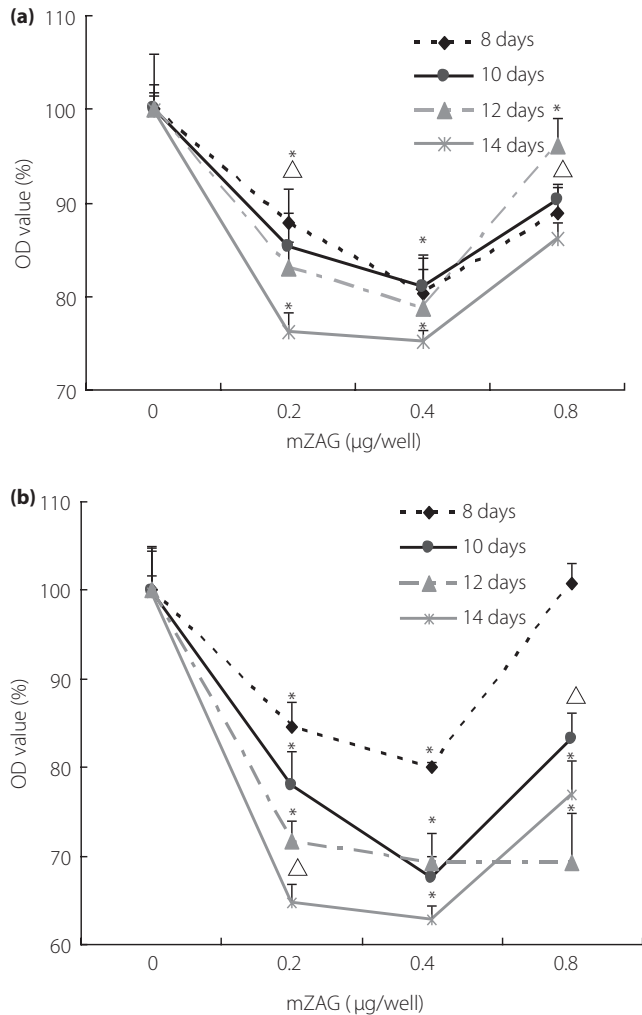
Figure 4 | The effect of zinc-α2-glycoprotein (ZAG) on the lipid accumulation of adipocytes as determined by the (a) Oil Red O staining method and (b) intracellular triglyceride (TG) content measurements. The 3T3-L1 cells were seeded in 24-well culture plates at a density of 2×10^4 cells per well. The cells were differentiated. On days 8, 10, 12 and 14 after differentiation, the murine ZAG (mZAG) expression plasmid (0.2, 0.4 or 0.8 μg/well, $n = 3$) was transfected into the adipocytes. A total of 48 h later, Oil Red O staining and the TG content assay were carried out. The data represent the mean ± standard error of the mean from three independent experiments. $^{\triangle}P < 0.05$, $^{*}P < 0.01$ compared with the control group (0 μg). OD, optical density.

Figure 5 | Zinc-α2-glycoprotein (ZAG) decreases the expression of peroxisome proliferators-activated receptor-γ (PPARγ), CCAAT enhancer-binding protein-α (C/EBPα) and the critical lipogenic gene, fatty acid synthase (FAS) in (a) differentiating (day 4) and (b) fully differentiated adipocytes (day 7). 3T3-L1 cells were seeded in 12-well plates at densities of 2×10^4 cells per well. The cells were differentiated. On days 4 and 7 after differentiation, the murine ZAG (mZAG) expression plasmid (0.4, 0.8 or 1.6 μg/well, $n = 3$) was transfected into the adipocytes. A total of 48 h later, the total ribonucleic acid was extracted and real-time reverse transcription polymerase chain reaction was carried out. All of the results were normalized to the values that were obtained for the 18S control, and the results are expressed as fold changes of the cycle threshold (C_t) value relative to the control value, which was defined as 100. The data represent the mean ± SEM from three independent experiments. $^{\triangle}P < 0.05$, $^{*}P < 0.01$, $^{**}P < 0.001$ compared with the control group (0 μg). RU, relative unit.

leads to an inhibition of preadipocyte differentiation. Further experiments are required to elucidate the details of the mechanism by which ZAG stimulates preadipocyte proliferation.

Adipocyte differentiation includes a series of programmed alterations in the expression of specific transcription factors including PPARγ, C/EBPα and sterol response element-binding proteins 1[25,26]. PPARγ and C/EBPα are known to be the most crucial transcription factors in adipogenesis and lipogenesis[27].

Ectopic expression of PPARγ is sufficient to induce C/EBPα and to fulfill the differentiation of the preadipocytes to mature adipocytes. Although the initial induction of C/EBPα is dependent on PPARγ, C/EBPα, in turn, is known to reinforce the expression of PPARγ and many specific genes required for adipocyte differentiation and lipogenesis. This positive feedback loop between PPARγ and C/EBPα is believed to be essential for the maintenance of adipocytes in the terminally differentiated
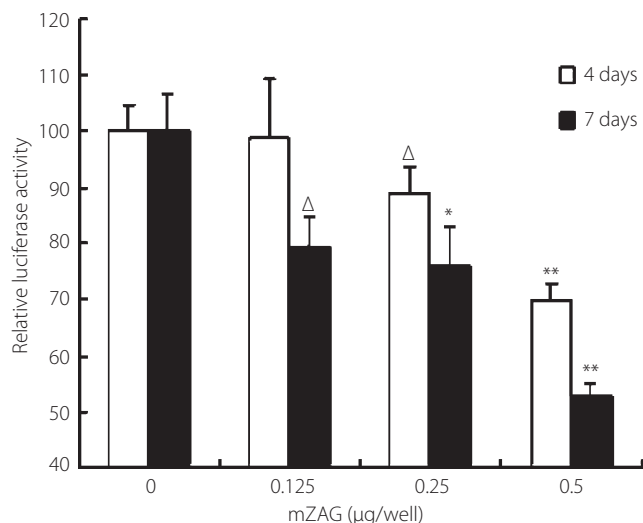
Figure 6 | Zinc-α2-glycoprotein (ZAG) suppresses fatty acid synthase (FAS) promoter activity in differentiating (day 4) and fully differentiated adipocytes (day 7). The 3T3-L1 cells were differentiated using a differentiation cocktail medium. On days 4 and 7 after differentiation, the murine ZAG (mZAG) plasmid (0.125, 0.25 or 0.5 μg/well; $n = 3$) was transfected together with a luciferase reporter gene plasmid hFAS625-Luc (0.5 μg/well) and an internal control plasmid pRL-SV40 (16.5 ng/well) into adipocytes using Lipofectamine 2000. After 48 h, the cells were lysed, and both the firefly and renilla luciferase activities were measured. The firefly luciferase activities were adjusted using the renilla luciferase activities. The data represent the mean ± standard error of the mean from three independent experiments. $^{\triangle}P < 0.05$, $^{*}P < 0.01$, $^{**}P < 0.001$ compared with the control group (0 μg).

state[28]. In the present study, the treatment adipocytes with ZAG significantly reduced the observed level of Oil-Red O staining and TG content, indicating a decrease in the number of lipid droplets in cytoplasm and a reduction in the size of adipocytes without the change of cell number, which has also been represented photographically (Figure 3). In order to investigate whether the reduced lipid accumulation in ZAG overexpressed cells was as a result of the inhibition of adipocyte differentiation, the expression of adipogenic markers in these cells was examined. The result showed that ZAG treatment significantly downregulated PPARγ and C/EBPα mRNA levels in the mid- and late-phase differentiation. Studies that were carried out by Akune *et al.* showed that PPARγ-deficient cells fail to differentiate into adipocytes, and that the overexpression of PPARγ and C/EBPα accelerates adipogenesis[29]. These results imply that ZAG suppresses adipocyte differentiation by inhibiting PPARγ and C/EBPα expression in the mid- and late-stages of adipocyte differentiation.

During mid-phase adipocyte differentiation, the expression of CEBPα and PPARγ increases, and then coordinately drive expression of adipocyte-specific genes, such as adipocyte, FAS, fatty acid-binding protein 2 and ACC, which characterize the final stages of differentiation. In the current study, ZAG expression notably suppressed FAS mRNA levels in differentiating and fully differentiated adipocytes, suggesting that ZAG inhibits lipogenesis through the downregulation of FAS expression. Furthermore, the results of the transient hFAS625-Luc plasmid transfection experiment showed that ZAG inhibited FAS expression by reducing FAS promoter activity. Our previous studies that were carried out in HFD-induced obese mice showed that the treatment of obese mice with ZAG significantly decreased the expression of key lipogenic enzymes, such as FAS, ACC and acyl-coenzyme A diacylglycerol transferase, in both adipose and liver tissue[5,15]. A similar result was reported in a very recent study by Choi *et al.*, who showed that small interference ribonucleic acid (siRNA)-mediated knock down of ZAG in liver cells led to a significant increase in the expression of lipogenic genes, including FAS and ACC[30]. All of these findings suggest that ZAG directly suppresses FAS expression, further inhibiting lipogenesis in adipose tissue and differentiated adipocytes. In addition, the reduction of lipid accumulation in ZAG overexpressed adipocytes might also be derived from the increase of lipolysis besides the inhibitory role of ZAG on lipogenesis. Previous studies both *in vivo* and *in vitro* have shown that ZAG stimulates lipid loss in adipose tissue and adipocytes by stimulating lipolysis through its interaction with b3-adrenoreceptors, suggesting a role of ZAG in lipid catabolism[7,31–34]. Overall, in the present study, we showed that ZAG, in addition to its role in promoting lipolysis, is involved in lipid metabolism by suppressing lipogenesis in adipose tissue and adipocytes[5]. These results, together with the recent finding that ZAG administration reduced fasting glucose and insulin levels in HFD-fed obese and *ob/ob* mice[5,12,35], suggest that ZAG could overcome certain metabolic alterations that are associated with the obese state and could be a promising target for anti-obesity and anti-diabetic therapies.

In conclusion, the present results show that ZAG promotes proliferation, and inhibits adipocyte differentiation and adipogenesis in 3T3-L1 cells. Furthermore, at the molecular level, ZAG downregulates the expression of PPARγ and C/EBPα, as well as the lipogenic-specific enzyme, FAS, by reducing FAS promoter activity. However, further molecular mechanism of ZAG on FAS expression remains to be elucidated.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 | Primers for amplifying target genes.