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# The Role of Adipsin, Complement Factor D, in the Pathogenesis of Graves' Orbitopathy

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**PURPOSE.** Graves' orbitopathy (GO) is an orbital manifestation of autoimmune Graves' disease, and orbital fibroblast is considered a target cell, producing pro-inflammatory cytokines and/or differentiating into adipocytes. Adipose tissue has been focused on as an endocrine and inflammatory organ secreting adipokines. We investigated the pathogenic role of a specific adipokine, adipsin, known as complement factor D in Graves' orbital fibroblasts.

**METHODS.** The messenger RNA (mRNA) expression of multiple adipokines was investigated in adipose tissues harvested from GO and healthy subjects. Adipsin protein production was analyzed in primary cultured orbital fibroblasts under insulin growth factor (IGF)-1, CD40 ligand (CD40L) stimulation, and adipogenesis. The effect of blocking *adipsin* with small interfering RNA (siRNA) on pro-inflammatory cytokine production and adipogenesis was evaluated using quantitative real-time PCR, Western blot, and ELISA. Adipogenic differentiation was identified using Oil Red O staining.

**RESULTS.** *Adipsin* gene expression was significantly elevated in GO tissue and increased after the stimulation of IGF-1 and CD40L, as well as adipocyte differentiation in GO cells. Silencing of *adipsin* suppressed IGF-1-induced *IL-6*, *IL-8*, *COX2*, *ICAM-1*, *CCL2* gene expression, and IL-6 protein secretion. Adipsin suppression also attenuated adipocyte differentiation. Exogenous treatment of recombinant adipsin resulted in the activation of the Akt, ERK, p-38, and JNK signaling pathways.

**CONCLUSIONS.** Adipsin, secreted by orbital fibroblasts, may play a distinct role in the pathogenesis of GO. Inhibition of adipsin ameliorated the production of pro-inflammatory cytokines and adipogenesis in orbital fibroblasts. Our study provides an in vitro basis suggesting adipsin as a potential therapeutic target for GO treatment.

**Keywords:** adipsin, Graves' orbitopathy (GO), inflammation, adipogenesis, orbital fibroblast

Graves orbitopathy (GO) is an autoimmune disease that occurs in half of the patients with Graves' disease (GD).<sup>1</sup> It is characterized by inflammation and expansion of orbital adipose tissue within bony orbit confines. In response to various stimuli, orbital fibroblasts produce excessive pro-inflammatory cytokines and/or differentiate into mature adipocytes.<sup>1</sup> Synergistic activation of the insulin-like growth factor (IGF)-1 receptor (IGF-1R) and the thyroid-stimulating hormone receptor (TSHR) has been identified in the pathogenesis of GO.<sup>2,3</sup> Moreover, orbital fibroblasts express CD40 and are activated by T cells via CD40/CD40L. Treatment of cells with CD154, a CD40 ligand (CD40L), upregulates the expression of pro-inflammatory cytokines and chemokines in Graves' orbital fibroblasts.<sup>4,5</sup>

The concept of adipose tissue as an inert storage depot has changed. Adipose tissue has been identified as an

endocrine and inflammatory organ that secretes adipokines that affect systemic metabolism. Adipokines overexpressed in autoimmune diseases, such as Crohn's disease and rheumatoid arthritis, have suggested a pathophysiologic role as pro-inflammatory cytokines.<sup>6-8</sup> Differentiated orbital pre-adipocyte fibroblasts are also known to express a high level of adipokines.<sup>9</sup> In addition, previous reports have shown that serum adipokine levels, including leptin, resistin, and allograft inflammatory factor-1 (AIF-1), were significantly associated with the presence of GO or elevated clinical activity scores (CAS), suggesting a role in the inflammatory process of GO.<sup>10,11</sup>

In this study, we initially examined transcript levels of adipokines in GO and normal orbital tissue, and, interestingly, *adipsin* was the highest elevated adipokine in GO tissue. Adipsin, a 24 kDa serine protease found in

3T3 adipocytes, was the first described adipokine in the late 1980s and was subsequently reported as a complement factor D (CFD).<sup>12-14</sup> Adipsin is predominantly secreted by adipocytes, but macrophages and monocytes can also synthesize adipsin.<sup>13</sup> The role of adipsin as a mediator interweaving metabolism and autoimmune diseases has been studied<sup>15,16</sup>; however, no studies to date have investigated adipsin in GO.

In light of the above, we aimed to investigate the pathogenic role of adipsin in the in vitro model of GO. Our study examined inhibition of adipsin effects on pro-inflammatory cytokine production and adipocyte differentiation, one of the main drivers of GO pathogenesis. Our findings suggest in vitro evidence implicating the role of adipsin in the inflammatory and adipogenic mechanism of GO and consideration of adipsin-targeted therapy as a treatment for GO.

## MATERIALS AND METHODS

### Reagents and Chemicals

The source of the reagents used in the study were as follows: Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12, 1:1), penicillin, and streptomycin (Welgene, Gyeongsan-si, Gyeongsangbuk-do, South Korea); fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Waltham, MA, USA); recombinant human IGF-1 (R&D Systems, Minneapolis, MN, USA); recombinant human CD40L (Enzo Life Sciences, Farmingdale, NY, USA); antibodies for C/EBP $\alpha$ , C/EBP $\beta$ , phosphorylated (p)-Akt, total (t)-Akt, p-extracellular signal-related kinase (ERK), t-ERK, p-p38, t-p38, p-c-Jun N-terminal kinases (JNK), t-JNK in Western blot (Cell Signaling Technology, Danvers, MA, USA); anti- $\beta$ -actin, peroxisome proliferator-activated receptor (PPAR)  $\gamma$ , adipsin, Acrp30 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and recombinant human adipsin protein (rhAdipsin; R&D Systems).

### Tissue and Cell Preparation

Orbital adipose tissues were collected from surgical byproducts during surgeries. GO tissues were harvested from orbital decompression surgery or lower lid blepharoplasty in 20 patients (14 women and 6 men, aged 21-67 years). All patients at the time of surgery had achieved euthyroid status and a CAS of 3 or less. Normal tissues were obtained in 12 patients (9 women and 3 men, aged 27-75 years) during upper or lower eyelid blepharoplasty with no history of GO or autoimmune thyroid disease (Supplementary Table S1). This study was approved by the institutional review board of Severance Hospital, Yonsei University College of Medicine (Seoul, Korea; IRB number: 4-2022-0999) and written informed consent was obtained from all the subjects after an explanation of the nature and possible consequences of the study. This study followed the tenets of the Declaration of Helsinki.

Tissue explants were minced and placed in DMEM/F12 containing 20% FBS, penicillin (100 U/mL), and streptomycin (100  $\mu$ g/mL) according to the previous study methods.<sup>17</sup> As orbital fibroblasts proliferated, the cells were passaged serially with trypsin/ethylenediaminetetraacetic acid (EDTA) and antibiotics and were incubated. Strains between the third and sixth passages were stored in liquid nitrogen and were used for subsequent experiments. The orbital fibro-

blasts were grown to confluence in 6 cm dishes, and the culture medium was changed to serum free DMEM/F12, followed by incubation with 10 ng/mL of IGF-1 or 100 ng/mL of CD40L.

### Silencing of Adipsin

Small interfering RNA (siRNA) designed to knock down the *adipsin* gene (*CFD*) and negative control #1 siRNA were obtained from Bioneer Corporation (Daedeokgu, Daejeon, South Korea; Supplementary Table S2). A concentration of 100 nM siRNAs was transfected into 80% confluent orbital fibroblasts with Lipofectamine 2000 (Invitrogen) according to the supplier's instructions. Six hours after transfection, the medium was changed to a fresh complete medium containing 10% FBS and antibiotics, and cells were maintained for 24 to 72 hours depending on the experiments.

### Western Blot

Orbital fibroblasts treated with each reagent were washed with cold phosphate-buffered saline (PBS) and then lysed in a cell lysis solution, as detailed previously.<sup>18</sup> Cell lysates were centrifuged, and protein concentrations in the supernatant of homogenous cell fractions were measured by BCA Kit (Thermo Fisher Scientific). Protein lysates were mixed with sample buffer, boiled, and loaded in 10% SDS-PAGE. Proteins were transferred to Nitrocellulose membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA), and incubated overnight with primary antibodies in Tris-buffered saline Tween 20 (TBST). Immunoreactive bands were identified using horseradish peroxidase-conjugated secondary antibodies. Then, the conjugated peroxidase was visualized by chemiluminescence (Thermo Fisher Scientific) and exposure to X-ray film (Agfa-Gevaert, Mortsel, Belgium). Each immunoreactive band was measured using densitometry and normalized to the amount of  $\beta$ -actin present in each sample.

### Real-Time Polymerase Chain Reaction

Orbital fat tissues, collected in RNAlater (Ambion, Austin, TX, USA) to avoid RNA degradation, were homogenized and lysed with a Precelly lysing kit (Bertin Instruments). Total RNA was extracted from the orbital fat tissue or IGF-1-treated orbital fibroblasts using TriZol (Invitrogen, Carlsbad, CA, USA). The quality of the RNA samples was checked using the spectrophotometric method with a microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA), and the values of A260/A280 were measured between 1.8 and 2.0. The cDNA was synthesized according to the manufacturer's instructions. PCR amplification was performed with specific primers and SYBR green PCR master mix with a QuantStudio3 real-time PCR thermocycler (Applied Biosystems, Carlsbad, CA, USA). The results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to account for PCR variability and represented as the fold change in cycle threshold (Ct) value in comparison to the control group, using the  $2^{-\Delta\Delta Ct}$  method. Primers specific for multiple adipokines that are related to autoimmune diseases or inflammation (*adipsin*, *adiponectin*, *resistin*, *chemerin*, *leptin*, *visfatin*, *omentin*, and *fatty acid-binding protein 4* [*FABP4*], *C1q/tumor necrosis factor* [*TNF*]-*related protein-3* [*CTRP3*] and *apelin*) and pro-inflammatory cytokines (*IL-6*, *IL-8*, *Cox-2*, *ICAM-1*, and *CCL2*) are shown in Supplementary Table S3.

## Enzyme-Linked Immunosorbent Assay

Orbital fibroblasts transfected with *adipsin*-targeting siRNA (si-*adipsin*) and negative control siRNA (si-con) were incubated for 48 and 72 hours, followed by IGF-1 treatment (24 hours, 10 ng/mL). Cell culture supernatants were analyzed for IL-6 using ELISA kits according to the manufacturer's instructions (R&D System, Minneapolis, MN, USA). The percentage of binding was measured by the absorbance of reactions measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

## Adipogenesis

Orbital pre-adipocyte fibroblasts were differentiated into adipocytes for 14 days using previously described methods.<sup>17,18</sup> To assess the effect of silencing *adipsin* on adipogenesis, cells were transfected with si-*adipsin* or si-con for 48 hours before 14-day differentiation period. Briefly, cells grown in 6-well plates were stimulated with adipogenic solutions; 10% FBS DMEM, 33  $\mu$ M biotin, 17  $\mu$ M pantothenic acid, 10  $\mu$ g/mL transferrin, 0.2 nM T3, 1  $\mu$ M insulin (Boehringer-Mannheim, Mannheim, Germany), 0.2  $\mu$ M carbaprostaglandin (cPGI2; Calbiochem, La Jolla, CA, USA), and 10  $\mu$ M rosiglitazone (Cayman, Ann Arbor, MI, USA). For the first 4 days, 10  $\mu$ M dexamethasone and 0.1 mM isobutylmethylxanthine (IBMX) were added in the media and the media was replaced every 2 to 3 days.

## Oil Red O Staining

To evaluate adipocyte differentiation, orbital fibroblasts were stained with Oil Red O as previously described.<sup>18</sup> In brief, cells were washed 2 times with 1  $\times$  PBS, fixed with 10% formalin in PBS at room temperature for 1 hour, and stained with 300  $\mu$ L Oil Red O working solution for 1 hour. The dishes were washed with distilled water and visualized using an Axiovert (Carl Zeiss, Jena, Germany) light microscope and photographed at  $\times 40$  or  $\times 100$  magnification with an Olympus BX60 light microscope (Olympus, Melville, NY, USA). To quantify lipid accumulation, Oil red O stain was solubilized with 100% isopropanol, and a spectrophotometer at 490 nm was used to measure the optical density of the solution.

## Statistical Analysis

For statistical analysis, IBM SPSS Statistics for Windows version 27.0 (IBM Corp., Armonk, NY, USA) was used. All experiments were duplicated on three samples from different patients, and the average was expressed as the mean values  $\pm$  standard deviation (SD). Depending on the normal distribution with Kolmogorov–Smirnov test, Student *t*-test, or Mann–Whitney *U* test was used to compare between the GO and healthy group or the experimental and control group. A *P* value less than 0.05 was assumed to be statistically significant.

## RESULTS

### mRNA Levels of Multiple Adipokines in Orbital Tissues

To investigate its potential role in GO, we measured the expression of adipokines in orbital tissues taken from subjects with GO ( $n = 20$ ) and healthy controls ( $n = 12$ ).

Of the 10 adipokines studied: *adipsin*, *adiponectin*, *apelin*, *chemerin*, *leptin*, *omentin*, *resistin*, *visfatin*, *FABP4*, and *CTRP3*; *adipsin* and *leptin* transcript levels were significantly greater in GO orbital tissues than in healthy control tissues ( $P < 0.05$ ). Especially, the basal level of *adipsin* mRNA was remarkably higher in GO tissues than in healthy tissues (median mRNA relative density, 1.54 in normal tissue versus 19.5 in GO tissue; Fig. 1).

### Adipsin Protein Elevation by IGF-1 and CD40 Ligand Stimulation in GO Orbital Fibroblasts

To assess the changes in *adipsin* expression levels in response to inflammatory stimulation, GO orbital fibroblasts were treated with IGF-1 and CD40L. IGF-1 (10 ng/mL) and CD40L (100 ng/mL) were applied to orbital fibroblasts for 0.5, 1, 6, and 24 hours, and *adipsin* protein levels measured by Western blot increased with a time-dependent manner ( $P < 0.05$ ; Fig. 2).

### Adipsin Elevation During Adipocyte Differentiation in Both GO and Normal Orbital Fibroblast

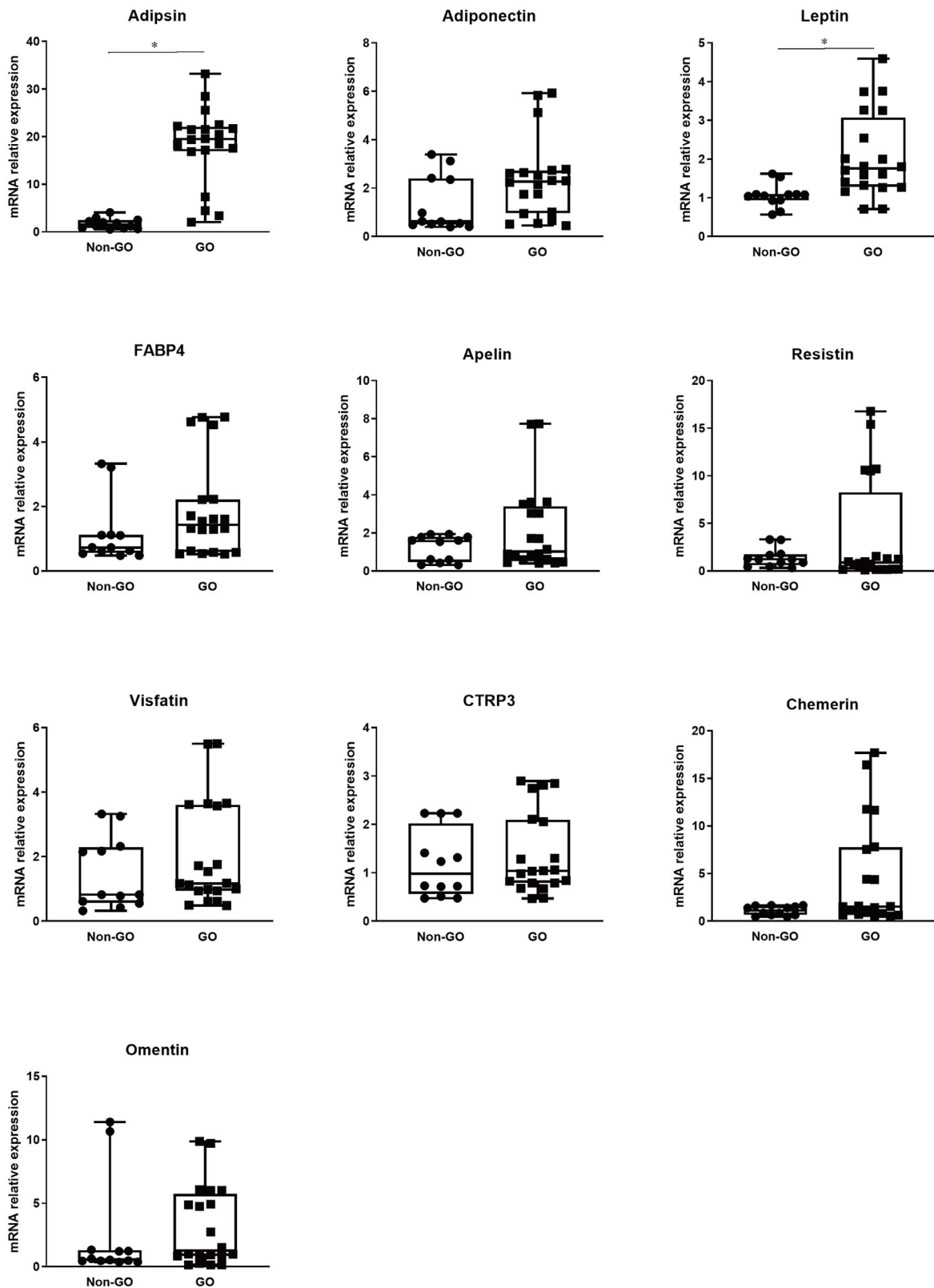
GO and normal orbital fibroblasts were differentiated into adipocytes under adipogenic stimulation for 14 days. Oil Red O staining showed increased size and number of intracytoplasmic lipid droplets (Fig. 3A) and optical density of stained cell lysates confirmed increasing adipocyte differentiation quantitatively ( $P < 0.05$ ; Fig. 3B). During adipogenesis, the level of *adipsin* transcripts were significantly upregulated especially in GO cells than normal cells (Fig. 3C). *Adipsin* protein expression levels were also elevated time-dependently during differentiation. Protein expression of adipogenic transcription factors, PPAR $\gamma$ , C/EBP $\alpha$ , and C/EBP $\beta$ , and mature adipocytes marker, *acrp30*, were increased after adipogenic differentiation of orbital fibroblasts (Fig. 3D).

### Knockdown of Adipsin Suppresses Pro-Inflammatory Cytokines Production

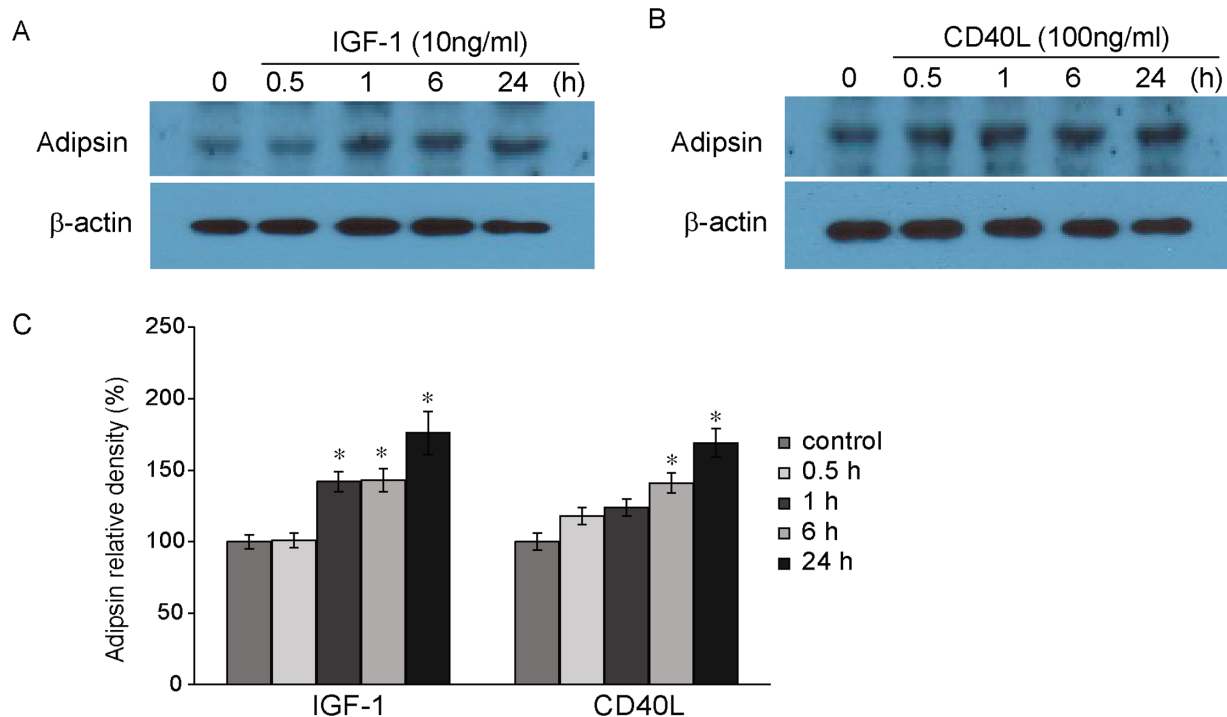
We verified that stimulation of orbital fibroblasts with IGF-1 (10 ng/mL for 24 hours) upregulated mRNA expression of pro-inflammatory cytokines, IL-6, IL-8, Cox-2, ICAM-1, and CCL2 and it peaked at 6 hours of treatment ( $P < 0.05$ ; Fig. 4A).

To clarify the role of *adipsin*, the *adipsin* gene was silenced via transfection of si-*adipsin*. The siRNA was transfected for 24 hours and then treated with IGF-1 (10 ng/mL) for 16 hours. GO and normal orbital fibroblasts showed silencing efficiency of si-*adipsin* compared with si-con using real-time PCR. Transcripts of proinflammatory cytokines induced by IGF-1 stimulation were significantly suppressed by *adipsin* silencing ( $P < 0.05$ ; Fig. 4B).

In addition, after siRNA transfection for 48 and 72 hours, IL-6 protein secretion was measured with ELISA. Increased release of IL-6 in response to challenge with 10 ng/mL IGF-1 for 24 hours was significantly blunted with si-*adipsin* transfection in both GO and normal cells ( $P < 0.05$ ; Fig. 5).



**FIGURE 1.** Expression of adipokine mRNA in GO and normal orbital tissues. GO and normal orbital tissues from GO ( $n = 20$ ) and healthy controls ( $n = 12$ ) were used to analyze the transcription level of multiple adipokines using real-time PCR. The analysis included 10 adipokines: *adipsin*, *adiponectin*, *apelin*, *chemerin*, *leptin*, *omentin*, *resistin*, *visfatin*, *FABP4*, and *CTRP3*. The graphs are presented as the median and interquartile ranges (\* $P < 0.05$  versus normal control tissues).



**FIGURE 2.** Expression of adipsin protein stimulated by IGF-1 and CD40L in GO orbital fibroblasts. GO orbital fibroblasts ( $n = 3$ ) were treated with IGF-1 (10 ng/mL) and CD40L (100 ng/mL) for increasing lengths of time (0–24 hours). Western blot analyses were performed at different time points (0, 0.5, 1, 6, and 24 hours) to investigate the protein expression of adipsin. Experiments were conducted in duplicate in individual cells from three different subjects. (A, B) Representative Western blot band of adipsin was shown. (C) The data in columns indicated the mean relative density ratio  $\pm$  SD using densitometry normalized to the level of  $\beta$ -actin in the same sample ( $*P < 0.05$ ).

### Knockdown of Adipsin Suppresses Adipogenesis

The effect of adipsin on adipogenesis in orbital fibroblasts was examined by silencing the *adipsin* gene. After transfection of si-adipsin or si-con for 48 hours, orbital fibroblasts were cultured in adipocyte differentiation medium for 14 days. Transfection of adipsin siRNA substantially reduced adipogenesis as identified with Oil Red O staining on day 14 of differentiation in GO orbital fibroblasts (Figs. 6A, 6B). In addition, adipsin suppression reduced protein expression of adipogenic transcription factors, C/EBP $\alpha$ , C/EBP $\beta$ , and acrp30 in GO orbital fibroblasts and C/EBP $\beta$  in normal orbital fibroblasts ( $P < 0.05$  versus si-con; Figs. 6C, 6D).

### Exogenous Recombinant Adipsin Upregulates Signaling Pathway Molecules

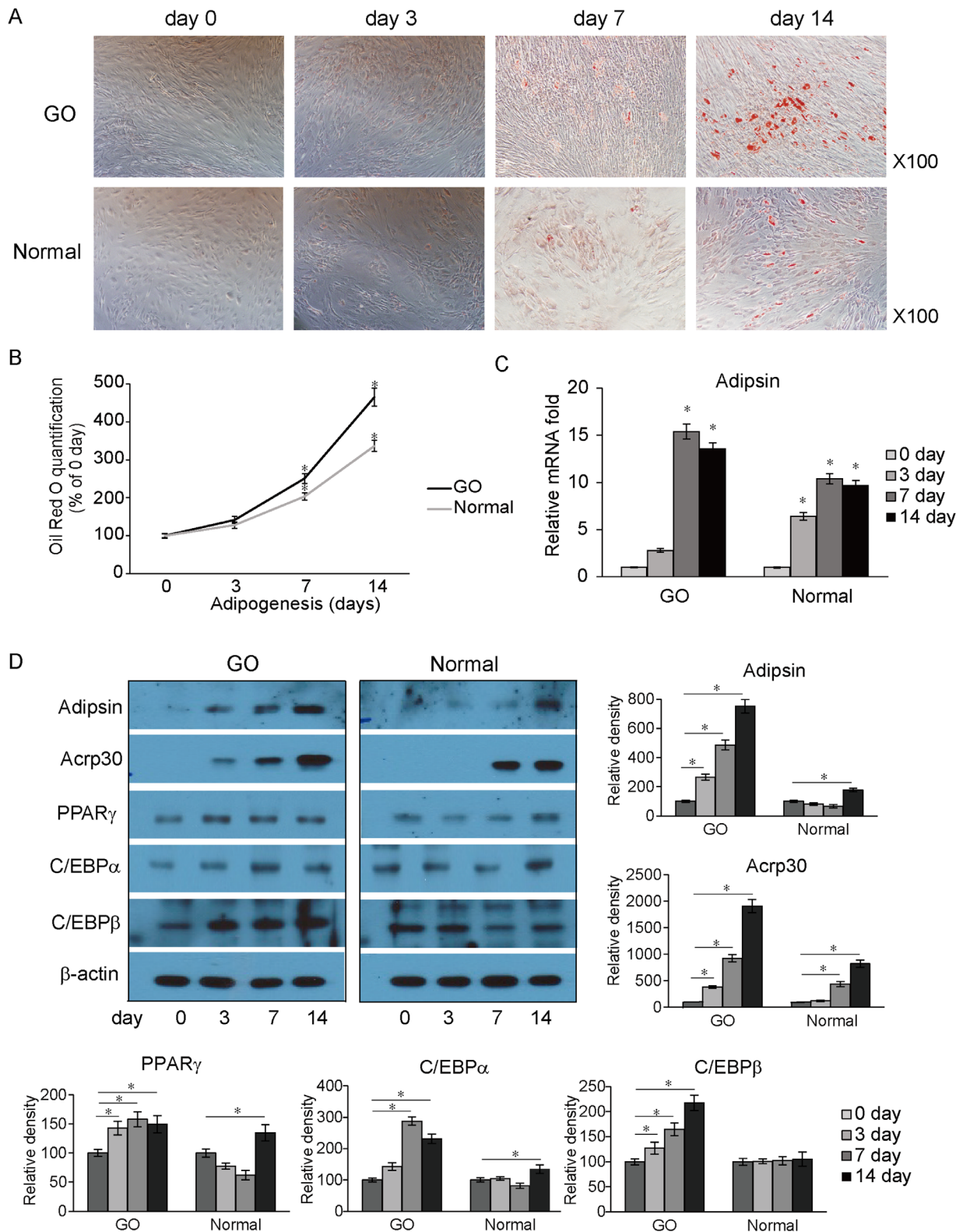
The rhAdipsin was added to orbital fibroblasts to identify the role of adipsin in the molecular signaling pathway of GO mechanism. Mitogen-activated protein kinase (MAPK) families consisting of ERK, JNK, p38, and phosphatidylinositol 3-kinase (PI3K)-Akt signaling cascade were investigated. GO and normal orbital fibroblasts were treated with rhAdipsin (10 ng/mL) for 1 hour and phosphorylated multiple transcription factors were measured at different time points (0, 15, 30, and 60 minutes) using Western blot analysis (Fig. 7A). In GO cells, Akt, ERK, and p38 phosphorylation increased after rhAdipsin treatment for 1 hour ( $P < 0.05$ ; Fig. 7B). In normal cells, ERK and p38 phosphorylation levels were elevated after 1 hour of rhAdipsin treatment ( $P < 0.05$ ; Fig. 7C). JNK was phosphorylated at a relatively early phase, 15

minutes, in both GO and normal orbital fibroblasts ( $P < 0.05$ ; see Figs. 7B, 7C).

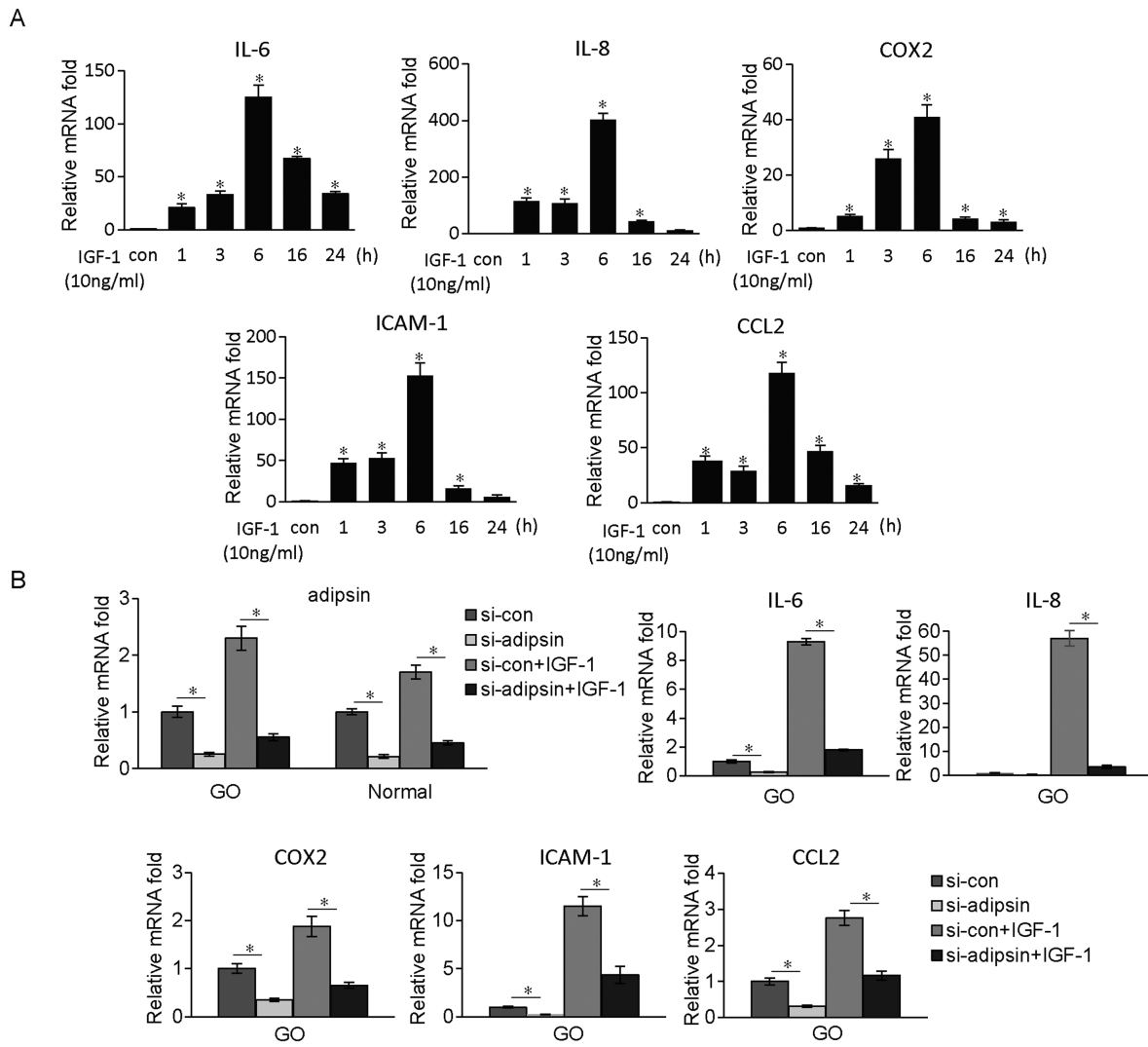
### DISCUSSION

In this study, multiple adipokines were assayed in orbital tissues and we found that adipsin ranked as the highest concentration in GO tissue compared to controls. We further investigated the role of adipsin in the inflammatory and adipogenic molecular pathways. This is the first study to demonstrate that adipsin production is increased in Graves' orbital tissue and orbital fibroblasts, and that blockage of adipsin ameliorated pro-inflammatory cytokine production and adipocyte differentiation *in vitro*.

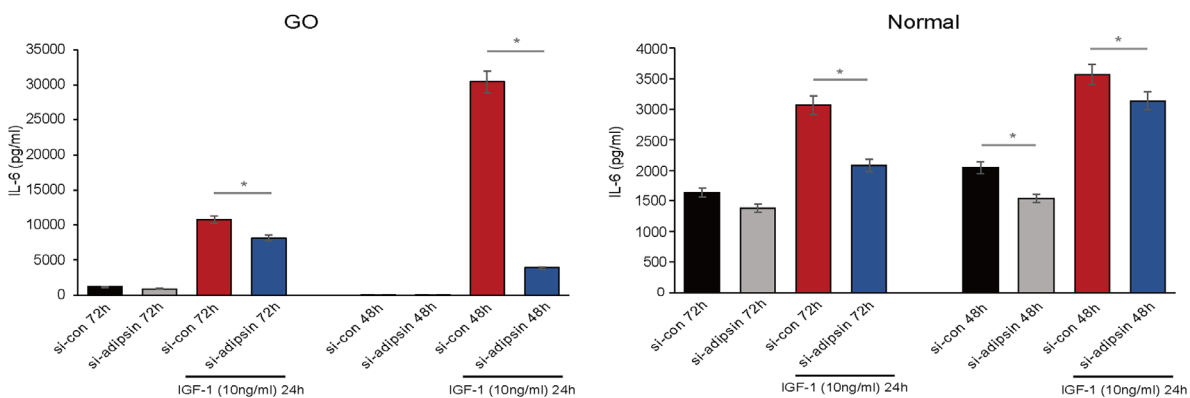
There is increasing evidence that adipogenesis is enhanced in the orbital tissue of patients with GO. Orbital fibroblasts are stimulated to undergo adipocyte differentiation with increased markers of adipocyte such as PPAR $\gamma$ , adiponectin, leptin, perilipin, and lipoprotein lipase.<sup>19–21</sup> Orbital fibroblasts or serum from patients with GD have been shown to express higher levels of adiponectin, leptin, resistin, and AIF-1.<sup>9–11</sup> In this study, among several elevated adipokines, *adipsin* mRNA expression level was noted to be the highest with GO tissue. IGF-1 and CD40L which upregulate pro-inflammatory cytokines, chemokines, and hyaluronan production in Graves' orbital fibroblasts<sup>4,5,22–24</sup> significantly increased the expression of adipsin levels in GO orbital fibroblasts. Silencing of adipsin also blocked IGF-1-induced pro-inflammatory cytokine expression, such as IL-6, IL-8, COX-2, ICAM-1, and CCL2. In particular, IL-6, an important pro-inflammatory molecule in GO pathogenesis which was stimulated by IGF-1, was significantly reduced



**FIGURE 3.** Adipsin induction during adipocyte differentiation in GO and normal orbital fibroblasts. Cells were cultured in an adipogenic medium for 14 days to induce adipogenesis. Experiments were performed duplicate in three different cells from different individual subjects and the graphs show mean ratios  $\pm$  SD relative to the undifferentiated day 0 orbital fibroblasts. (A) Orbital fibroblasts stained with Oil Red O solution represented lipid accumulation after initiation of 14-day adipogenesis and were photographed at  $\times 100$  magnification. (B) To quantitative assessment, optical density of solubilized Oil Red O staining was measured at 490 nm ( $*P < 0.05$ ). (C) Adipsin mRNA expression level was determined by real-time PCR ( $*P < 0.05$ ). (D) Western blot analyses showed increased production of adipsin, acrp30 and adipogenic transcription factors, PPAR $\gamma$ , C/EBP $\alpha$ , and C/EBP $\beta$  during adipogenesis. The results from orbital fibroblasts are presented as the mean density ratio  $\pm$  SD, normalized to the level of  $\beta$ -actin in the same sample ( $*P < 0.05$  versus control cells).

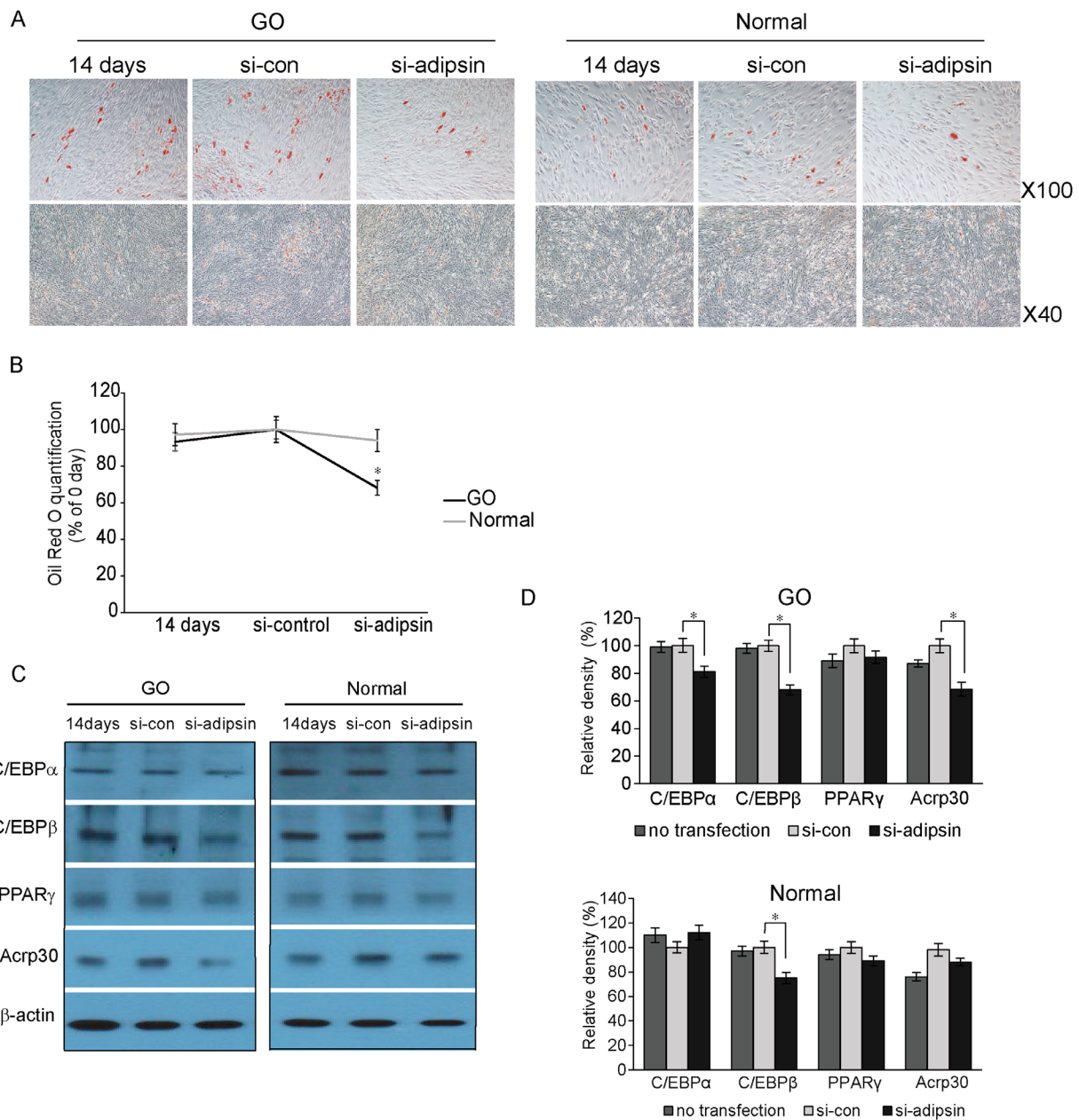


**FIGURE 4.** Effect of adipsin knockdown on IGF-1 induced mRNA expression of proinflammatory cytokines in orbital fibroblasts. (A) IGF-1 (10 ng/mL) was exposed to GO orbital fibroblasts ( $n = 3$ ) with a time-dependent manner for 1, 3, 6, 16, and 24 hours, and mRNA of pro-inflammatory cytokines, IL-6, IL-8, Cox-2, ICAM-1, and CCL2, were measured using real-time PCR ( $*P < 0.05$ ). (B) GO ( $n = 3$ ) and normal ( $n = 3$ ) orbital fibroblasts were transfected with siRNA targeting adipsin (si-adipsin) or negative control (si-con) for 24 hours and exposed to IGF-1 (10 ng/mL) for 16 hours ( $*P < 0.05$ ). Data in columns indicate the mean relative fold  $\pm$  SD of mRNA level compared to the control.



**FIGURE 5.** Suppressive effect of adipsin knockdown on IL-6 protein secretion by ELISA. GO ( $n = 3$ ) and normal ( $n = 3$ ) orbital fibroblasts were transfected with siRNA targeting adipsin (si-adipsin) or control-siRNA (si-con) for 48 and 72 hours, followed by treating 10 ng/mL of IGF-1 for 24 hours. IL-6 protein secretion was quantified with ELISA in GO (A) and normal (B) orbital fibroblasts. Experiments were performed duplicate in three different cells from different individual samples and the mean value  $\pm$  SD was represented as column ( $*P < 0.05$ , si-con versus si-adipsin).





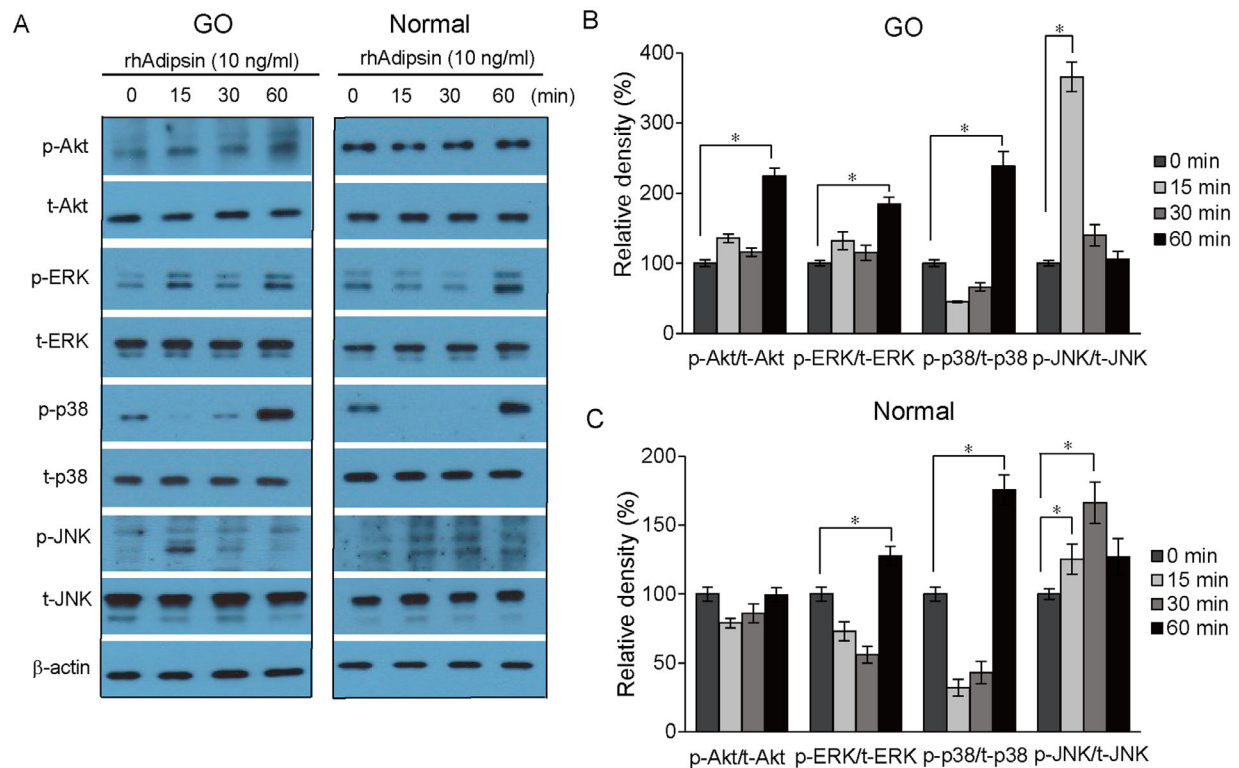
**FIGURE 6.** Suppressive effect of silencing adipsin on adipocyte differentiation. Orbital fibroblasts from patients with GO ( $n = 3$ ) were incubated with adipogenic medium for 14 days with or without pretreatment of transfecting si-con or si-adipsin for 48 hours. (A) Orbital fibroblasts were stained with Oil Red O and examined using microscope  $\times 40$  and  $\times 100$  magnifications. (B) Cell-bound Oil Red O was solubilized, and optical density at 490 nm was measured to quantify the adipogenesis. Each data was represented as the mean relative density compared to si-control cells. (C) Adipogenic transcription factors, PPAR $\gamma$ , C/EBP $\alpha$ , C/EBP $\beta$ , and acrp30, were analyzed by Western blotting and representative gel images are shown. (D) The results are shown as relative mean density  $\pm$  SD normalized to the level of  $\beta$ -actin in the same sample, respectively ( $*P < 0.05$  versus si-control cells).

by adipsin blockade, suggesting that adipsin may influence the downstream pathway of IGF-1/IGF-1R in GO.

Adipsin is previously known to promote new adipocyte formation and affects adipose tissue remodeling. *Adipsin* knockout in mice inhibits bone marrow adipogenesis<sup>25</sup> and overexpression of the *adipsin* gene in 3T3-L1 cells increases adipocyte differentiation.<sup>26</sup> Adipsin serum concentrations are strongly related to obesity and age.<sup>27</sup> Orbital fibroblasts in GO are considered to have a higher potential to differentiate into mature adipocytes and exhibit exaggerated responses to inflammatory stimuli. Infiltration

of Graves' orbital tissue with autoreactive T cells and macrophages leads to the profound secretion of pro-inflammatory molecules, which triggers the adipogenic conversion of orbital fibroblasts. We have found that adipsin expression markedly increases after adipogenic stimuli in Graves' orbital fibroblasts and its silencing suppresses adipogenesis as well as pro-inflammatory cytokine production, suggesting a novel potential therapeutic target of the inflammatory and adipogenic mechanism of GO.

Several papers have demonstrated the relationship of the inflammatory mechanism of adipsin in autoimmune



**FIGURE 7.** Signal pathway molecules after treating orbital fibroblasts with rhAdipsin. GO ( $n = 3$ ) and normal ( $n = 3$ ) orbital fibroblasts were treated rhAdipsin for 0, 15, 30, and 60 minutes. Phosphorylated and total Akt, ERK, p38, and JNK were determined using Western blot analysis. Tests were duplicated in three different cells from different subjects. (A) Representative bands from Western blot analyses are shown. (B, C) Data in the columns indicate the mean density ratio  $\pm$  SD by densitometry normalized to the level of  $\beta$ -actin in the same sample and represent relatively to non-treated (0 min). Differences between control cells and rhAdipsin treated cells are indicated ( $*P < 0.05$  versus control).

diseases, including rheumatoid arthritis (RA),<sup>28</sup> systemic lupus erythematosus (SLE),<sup>29</sup> systemic sclerosis,<sup>30</sup> and multiple sclerosis.<sup>31</sup> Depletion of adipsin in fat-free mice prevented inflammatory arthritis by modifying the infiltration of neutrophils.<sup>16</sup> Adipsin-deficient mice also showed decreased disease activity and renal injury in SLE.<sup>15</sup> These studies are in line with our results that inhibiting adipsin reduces pro-inflammatory cytokine production.

Stimulation of orbital fibroblasts with rhAdipsin elevates ERK, p38, and JNK phosphorylation in both GO and normal orbital fibroblasts, and Akt phosphorylation is enhanced only in GO cells. PI3K-Akt signaling cascade has been verified to play a role in GO adipogenesis and inflammation in several studies.<sup>32,33</sup> Especially in adipogenesis, phosphorylation of Akt activates forkhead box protein O1 (FOXO1), leading to an increase in adipogenic transcription factors.<sup>33–35</sup> Further, recent studies have discovered that PI3K-Akt signaling serves as a downstream pathway of IGF-1R, as well as TSHR.<sup>36–38</sup> Taken together, IGF-1-induced adipsin could play a role in inciting further inflammation or adipogenesis via the Akt signaling cascade.

Meanwhile, there is a need to further investigate the role of adipsin in GO pathogenesis and its connection to the complement system. After adipsin was identified as CFD, the role of the complement system in adipocyte metabolism has been extensively studied.<sup>39</sup> Notably, because CFD plays a crucial role in the alternative complement pathway, promoting the production of C3a, Song et al. demonstrated that CFD stimulates adipogenesis and lipid accu-

mulation in preadipocytes through the C3a-C3a receptor pathway.<sup>26</sup> Furthermore, dysregulated complement activation can lead to excessive inflammation potentially resulting in autoimmune disorders or systemic inflammation.<sup>40,41</sup> In line with this, Bai et al. have found elevated expression of the C5 gene in GO orbital adipose tissue through transcriptome analysis.<sup>42</sup> The specific mechanism through which blocking adipsin exerts a protective role in GO remains unclear from the current studies. Although we speculate that inhibiting the formation of inflammatory mediators, such as C3a and C5a, could be one mechanism, it is important to acknowledge that other mechanisms may also be at play. To shed light on this, performing single-cell RNA sequencing on orbital fibroblasts with silenced or upregulated adipsin could be instrumental in analyzing the underlying mechanism. In addition, adipsin needs to be cleaved by the mannose-binding lectin-associated serine protease-3 (MASP-3) to become fully activated.<sup>43</sup> In an RA mouse model, inhibiting MASP-3 using siRNA alleviated the clinical disease activity.<sup>44</sup> Hence, further investigations concerning the concentration or functional role of MASP-3 in GO are also warranted to better understand the pathogenic mechanism of adipsin in GO.

Complement system including CFD has a function in both physiologic processes and autoimmune diseases. Blockage of certain aspects possibly prevents systemic inflammation and disease progression.<sup>41,45</sup> Monoclonal blocking antibodies for CFD, as well as small molecules, have been developed and have become potential orphan drugs in

the field of complement-mediated diseases, such as paroxysmal nocturnal hemoglobinuria (PNH), cold agglutinin disease, and transplant-associated thrombomicroangiopathy.<sup>41,46</sup> Small molecules targeting CFD inhibiting cleavage of factor B reduced PNH cell hemolysis and blocked a dysregulated alternative complement pathway in a typical hemolytic uremic syndrome in vitro.<sup>47</sup> A first-in-class oral small molecule inhibitor of CFD, danicopan, inhibited intravascular hemolysis and increased hemoglobin concentration in a phase II trial for untreated patients with PNH.<sup>48</sup> It is also in a current phase III (NCT04469465) trial as an add-on therapy to a C5 inhibitor. Intravitreal injection of a humanized monoclonal antibody against CFD, lapalizumab, inhibited the ocular alternative complement pathway, although it did fail to halt geographic atrophy secondary to age-related macular degeneration in phase III clinical trial.<sup>49,50</sup>

Some patients with coronavirus disease 2019 (COVID-19) exhibit similar clinical characteristics to those with complement-mediated disorders, including thromboinflammation and endotheliopathy.<sup>51</sup> Jia Yu et al. reported the COVID-19 spike protein activated the alternative complement pathway directly, and anti-C5 antibody and CFD inhibitor (ACH145951) reduced the complement activation induced by the serum of patients with COVID-19.<sup>52,53</sup> Based on the emerging evidence for the role of complement in COVID-19, a phase II study evaluating the treatment efficacy of remdesivir with or without danicopan was recently completed (NCT04988035). After the COVID-19 infection, new-onset or worsening of GO was sometimes observed in clinical practices.<sup>54</sup> Although the role of the new complement inhibitory drugs in autoimmune diseases is not yet clear, it would be interesting to find whether inhibition of CFD could be a treatment option for GO through a clinical validation study.

In conclusion, our findings reveal for the first time that adipsin has a pathogenic role in adipogenesis and inflammation in the GO orbital fibroblast. Adipsin expression is increased in GO orbital tissues and IGF-1-induced orbital fibroblasts. Silencing adipsin attenuates adipocyte differentiation and pro-inflammatory cytokine production, suggesting that adipsin could be a promising therapeutic target for GO. Prior to developing potential therapeutics, further underlying mechanisms of adipsin need to be elucidated both in vitro and in vivo.

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