

Growth arrest-specific 6 modulates adiponectin expression and insulin resistance in adipose tissue

Sheng-Chiang Su¹, Chi-Fu Chiang² , Chang-Hsun Hsieh¹, Giieh-Hua Lu¹, Jih-Syuan Liu¹, Yi-Shing Shieh^{2,3,4}, Yi-Jen Hung^{1,4}, Chien-Hsing Lee^{1,4*}

¹Division of Endocrinology and Metabolism, Department of Internal Medicine, National Defense Medical Center, Tri-Service General Hospital, Taipei, Taiwan, ²School of Dentistry, National Defense Medical Center, Taipei, Taiwan, ³Department of Oral Diagnosis and Pathology, National Defense Medical Center, Tri-Service General Hospital, Taipei, Taiwan, and ⁴Division of Biochemistry, National Defense Medical Center, Taipei, Taiwan

Keywords

Adipocyte, Adiponectin, Gas6

*Correspondence

Chien-Hsing Lee
Tel: +886-2-87927182
Fax: +886-2-87927183
E-mail address:
doc10383@gmail.com

J Diabetes Investig 2021; 12: 485–492

doi: 10.1111/jdi.13412

ABSTRACT

Aims/Introduction: Obesity is characterized by disturbed adipocytokine expression and insulin resistance in adipocytes. Growth arrest-specific 6 (*GAS6*) is a gene encoding the Gas6 protein, which is expressed in fibroblasts, and its related signaling might be associated with adipose tissue inflammation, glucose intolerance and insulin resistance. The aim of this study was to investigate the associations among Gas6, adipocytokines and insulin resistance in adipocytes.

Materials and Methods: Mature Simpson Golabi Behmel Syndrome adipocytes were treated with high levels of insulin to mimic insulin resistance, and were examined for the expressions of Gas6, cytokines and adipocytokines from preadipocytes in differentiation. In an animal study, high-fat diet-induced obese mice were used to verify the Gas6 expression *in vitro*.

Results: During the differentiation of adipocytes, the expression of Gas6 gradually decreased, and was obviously downregulated with adipocyte inflammation and insulin resistance. Gas6 levels were found to be in proportion to the expression of adiponectin, which has been regarded as closely relevant to improved insulin sensitivity after metformin treatment. Similar results were also confirmed in the animal study.

Conclusions: Our results suggest that Gas6 might modulate the expression of adiponectin, and might therefore be associated with insulin resistance in adipose tissues.

INTRODUCTION

Obesity and its comorbidities, including diabetes mellitus, insulin resistance and cardiovascular diseases, are major global health issues¹. Over the past decade, there is increasing evidence to indicate inflammation links between obesity, diabetes and metabolic disease clusters². The inflammation caused by obesity is characterized by hypertrophy of adipocytes, accompanied by angiogenesis and immune cell infiltration, resulting in overproduction and prolongation of pro-inflammatory adipocytokines³. In addition, adipose tissue has been shown to secrete various adipokines, which exert biological roles in specific manners, to put remarkable influence on pathophysiological progression during the development of obesity-related complication³.

The TAM receptors (Tyro3, Axl and MerTK) play a pivotal role in innate immunity, and their ligands are growth arrest-

specific protein 6 (Gas6), a secreted vitamin K-dependent protein⁴. The *GAS6* gene was originally identified in fibroblasts, and it has been shown to be increased expression due to serum starvation and contact inhibition⁵. Therefore, Gas6 protein is also associated with survival, proliferation, cell adhesion and hemostasis, and is found in many tissues and various cells, especially in adipocytes⁵. Several studies have identified a link between Gas6, adipose tissue development and recruited macrophages into adipose tissue *in vitro* and *in vivo*^{6,7}, suggesting that Gas6/TAM plays an important role in the pathogenesis of adipose tissue inflammation. In addition, preclinical research has shown that Gas6 might regulate obesity-related inflammation and insulin resistance⁷. However, the clinical significance of Gas6 in human adipose tissue inflammation and insulin resistance has yet to be investigated.

Metformin, a drug for treatment of obesity and diabetes mellitus has been shown to ameliorate insulin resistance⁸. Recent

Received 17 October 2019; revised 30 July 2020; accepted 13 September 2020

studies have shown that metformin has a potential role in regulating adipose tissue inflammation⁹. Therefore, in the present study, we investigated whether the *GAS6* gene modulates adiponectin secretion in adipocytes, and in turn whether this mitigates adipose tissue insulin resistance. Furthermore, we investigated whether metformin enhances *Gas6* and adiponectin expressions, and thereby regulates adipocyte insulin resistance.

METHODS

Differentiation of human preadipocytes *in vitro*

Human Simpson Golabi Behmel Syndrome was kindly provided by the Graduate Institute of Physiology, National Defense Medical Center, Taipei, Taiwan. The Simpson Golabi Behmel Syndrome adipocytes growth and differentiation were carried out using the standard protocol¹⁰. Typically, 5,000 cells/well are grown in six-well plates for 3 days to near confluence in Dulbecco's modified Eagle's medium/nutrient mixture F-12 containing 10% fetal calf serum. When the cells reach 80% confluence, the differentiation process is started (day 0) by washing cells three times with phosphate-buffered saline (PBS) and then changing to a serum-free differentiation medium (Dulbecco's modified Eagle's medium/nutrient mixture F-12 supplemented with 10 µg/mL apo-transferrin, 20 nmol/L human recombinant insulin, 10 nmol/L hydrocortisone and 0.2 nmol/L tri-iodothyroxine). After 4 days, medium is changed, and cells are further cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 supplemented with 25 nmol/L dexamethasone, 500 µmol/L isobutyl-1-methylxanthine and 2 µmol/L thiazolidinedione (TZD).

Oil Red O staining assay

To measure the cell oil droplet content, the cells were stained with Oil Red O, rinsed with PBS and soaked in 10% formalin, then stained with filtered 0.1% Oil Red O solution (Oil Red O stock solution: dH₂O = 3:2), rinsed with PBS and dried. Finally, the stained cells were observed under a microscope. Quantification was carried out with 100% isopropanol and measured at 500 nm using a spectrophotometer.

Quantitative polymerase chain reaction analysis

Ribonucleic acid (RNA) was extracted using Trizol (Invitrogen, Oshima, NY, USA), followed by complementary DNA synthesis using a SuperScript[®] III Reverse Transcriptase kit (Thermo, Wilmington, DE, USA). Then, SYBR green dye (Thermo) was separately added with the primer of the target genes. The primer sequences are listed in Table 1. Two-step polymerase chain reaction using LC480 system (Roche, San Francisco, CA, USA) was preheated at 95°C for 7 min and 40 cycles, each cycle was set at denaturation step at 95°C for 10 s, and annealing step at 60°C for 30 s. The ratio of the target genes normalized to the internal standard (glyceraldehyde 3-phosphate dehydrogenase).

Enzyme-linked immunosorbent assay

Expressions of *Gas6* and adiponectin were determined using a DuoSet ELISA kit (R&D, Minneapolis, MN, USA) according to

the manufacturer's instructions. The following factors prepared at 50 and 0.891 ng/mL were assayed, and showed no cross-reactivity or interference. Each sample was tested in duplicate.

Western blotting

Proteins were extracted with lysis buffer containing radioimmunoprecipitation assay buffer, protease inhibitor (Thermo) and phosphatase inhibitor (Thermo) for 30 min. Protein concentration was quantified by bicinchoninic acid assay (Thermo, Rockford, IL, USA) and bovine serum albumin was used as standard protein. Equal amounts of proteins were loaded into 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrophoretically separated and subsequently transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked by using 5% non-fat milk in 0.01% Tris-buffered saline Tween 20 for 1 h at room temperature and subsequently probed overnight at 4°C with specific primary antibodies: *Gas6* (G307; Bioworld, Minneapolis, MN, USA), adenosine monophosphate-activated protein kinase (AMPK; 3694-S; Abcam, Cambridge, UK), phospho-AMPK (2802-S; Abcam), phospho-IRS-1 (Ser307; 2381; Cell Signaling, Danvers, MA, USA), IRS-1 (3407; Cell Signaling), phospho-Akt (9271; Cell Signaling), Akt (9272; Cell Signaling) and

Table 1 | Primer used for quantitative polymerase chain reaction analysis

Genes	Primer sequence (5' → 3')
IRS-1	
Forward primer	GCAACCAGAGTGCCAAAGTAG
Reverse primer	GGAGAAAGTCTCGGAGCTATGC
FASN	
Forward primer	CGGACATGGAGCACAAACAGG
Reverse primer	GCCATGGTACTTGGCCCTTGG
PPAR-γ	
Forward primer	ACCCAGAAAGCGATTCCCTCAC
Reverse primer	CCACGGAGCTGATCCCAAAG
<i>Gas6</i>	
Forward primer	CTCGTGAGCCTATAAACCTT
Reverse primer	TCCTCGTTCACITTCACCG
Adiponectin	
Forward primer	AAGGAGATCCAGGTCTTATTGG
Reverse primer	ACCTTACAGCCCCGGGTAC
Leptin	
Forward primer	TTTGCCCTATCTTTTCTATGTCC
Reverse primer	TGGAGGAGACTGACTGCGTG
Resistin	
Forward primer	GCTGTTGGTGTCTAGCAAGAC
Reverse primer	CATCATCATCATCTCCAG
RBP4	
Forward primer	GCCTCTTCTGCAGGACAAC
Reverse primer	GCACACGTCCCAGTATTCA
GAPDH	
Forward primer	CCACATCGCTCAGACACCAT
Reverse primer	TGACCAGGCCCAATA

glyceraldehyde 3-phosphate dehydrogenase (ab8245; Abcam). The next day, the membranes were washed with 0.01% Tris-buffered saline Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The reacted proteins were detected by chemiluminescence ECL kit (Merck Millipore, Darmstadt, Germany) and camera image systems.

Lentiviral short hairpin RNA transfection and adenoviral infection

Lentiviral-based Gas6 and control short hairpin RNA were obtained from the RNAi core of Academia Sinica, Taipei, Taiwan. The transfection process was based on experimental protocol¹¹.

Animal experiments

C57BL/6J mice were provided from the National Laboratory Animal Center, bred in-house and following the guidelines of the Laboratory Animal Center of the National Defense Medical Center. At 8 weeks-of-age, male C57BL/6J mice were fed a high-fat diet (60% kcals from fat) or a chow diet (10% kcals from fat) for 16 weeks ($n = 10$ in each group). After the final administration, mice were fasted for 6 h, then arterial blood was collected, and then plasma was obtained by centrifugation (120 g, 15 min) at room temperature and stored at -20°C . Biochemical analysis measures fasting plasma glucose and triglycerides. Homeostasis model of assessment of insulin resistance = glucose level \times serum insulin level / 22.5^{12} .

Immunohistochemistry assay

Immunohistochemistry was carried out on 3 m-thick paraffin embedded slides of fat depots. After deparaffinization and rehydration, the slides were brought to a boil in 10 mmol/L sodium citrate buffer pH 6.0, then maintained at a sub-boiling temperature for 10 min. Thereafter, the slides were cooled on a bench top for 30 min, and subsequently, they were treated with dH_2O for three times for 5 min each for washing. After incubation in 3% hydrogen peroxide for 10 min and washing again, those slides were rinsed with PBS and incubated in a 100- μL blocking solution for 1 h at room temperature. Thereafter, the tissue slides were then incubated at 4°C overnight with the following primary antibodies: Gas6 (G307; Bioworld) and adiponectin (AHP1005; AbD Serotec, Hercules, CA, USA). After washing in Tris-buffered saline Tween 20 three times, the slides were incubated for 1 h with specific secondary antibodies at room temperature. Signals were visualized by an improved chemiluminescence assay, and the tissues were photographed by Inverted Fluorescence Microscope (Leica, Wetzlar, Germany).

Statistical analysis

Results are expressed as the mean \pm standard error of the mean. Student's *t*-test with a two-tailed distribution was carried out to test the statistical significance between the two groups. Statistical significance was calculated using GraphPad Prism

6.01 software (GraphPad, La Jolla, CA, USA). Statistical significance was considered to be at 5% alpha-error levels ($P < 0.05$).

RESULTS

Expression of Gas6 at different stages of adipogenesis

To quantify the differentiation efficiency and insulin resistance state, we used human preadipocytes (day 0) that had differentiated into mature adipocytes (day 7 and day 14). These cells were treated with high insulin (1 $\mu\text{mol/L}$) for 48 h to mimic insulin resistance conditions (day 16). Subsequently, we carried out Oil Red O staining assay, and examined insulin resistance and adipogenic markers (insulin receptor substrate, tumor necrosis factor- α , fatty acid synthase and peroxisome proliferator-activated receptor gamma). Oil Red O staining showed the gradual appearance of lipid droplets as the human preadipocytes matured, and accounted for $>60\%$ of the adipocytes at day 16 while they were in a hyperinsulinemic state (Figure 1a). Both the expressions of insulin resistance markers (insulin receptor substrate, tumor necrosis factor- α) and adipogenic markers (fatty acid synthase, peroxisome proliferator-activated receptor gamma) progressively increased during the differentiation of the preadipocytes (Figure 1b; P for trend <0.01), with the highest expressions noted in the adipocytes at day 16 (Figure 1b). To investigate the potential roles of Gas6 during the differentiation of the adipocytes, we isolated both Gas6 messenger RNA and protein from the adipocytes. The results showed that the expression of Gas6 was higher in the adipocytes at day 0 and day 7, but lower at day 16 (Figure 2a,b; P for trend <0.01). Similar results were also found for the expression of circulating Gas6 protein by enzyme-linked immunosorbent assay (Figure 2c).

Gas6 modulated the expression of adiponectin in adipocytes

Previous studies have shown that many kinds of adipocytokines are released during the differentiation and inflammation of adipocytes. Therefore, we used effective shGas6 knockdown constructs in day-7 adipocytes (Figure 3a), and then examined the adipocytokine gene expressions (adiponectin, leptin, resistin and retinol-binding protein 4) in human adipocytes after successful delivery of shGas6. The adiponectin expressions in messenger RNA and protein were decreased when Gas6 knockdown was established in day-7 adipocytes (Figure 3b,c), suggesting that Gas6 facilitated the expression of adiponectin in the adipocytes.

Expression of Gas6 and adiponectin in insulin-resistant obese mice

To evaluate the expressions of Gas6 and adiponectin, the mice were fed with a high-fat diet (60% fat for 16 weeks) to induce obesity to mimic obesity and insulin resistance conditions. The average bodyweight, glucose level, homeostasis model of assessment of insulin resistance and triglyceride level were increased in these mice (Figure 4a). The expressions of Gas6 and adiponectin in the adipocyte tissue of these animals and normal controls were then analyzed by immunohistochemistry. In the

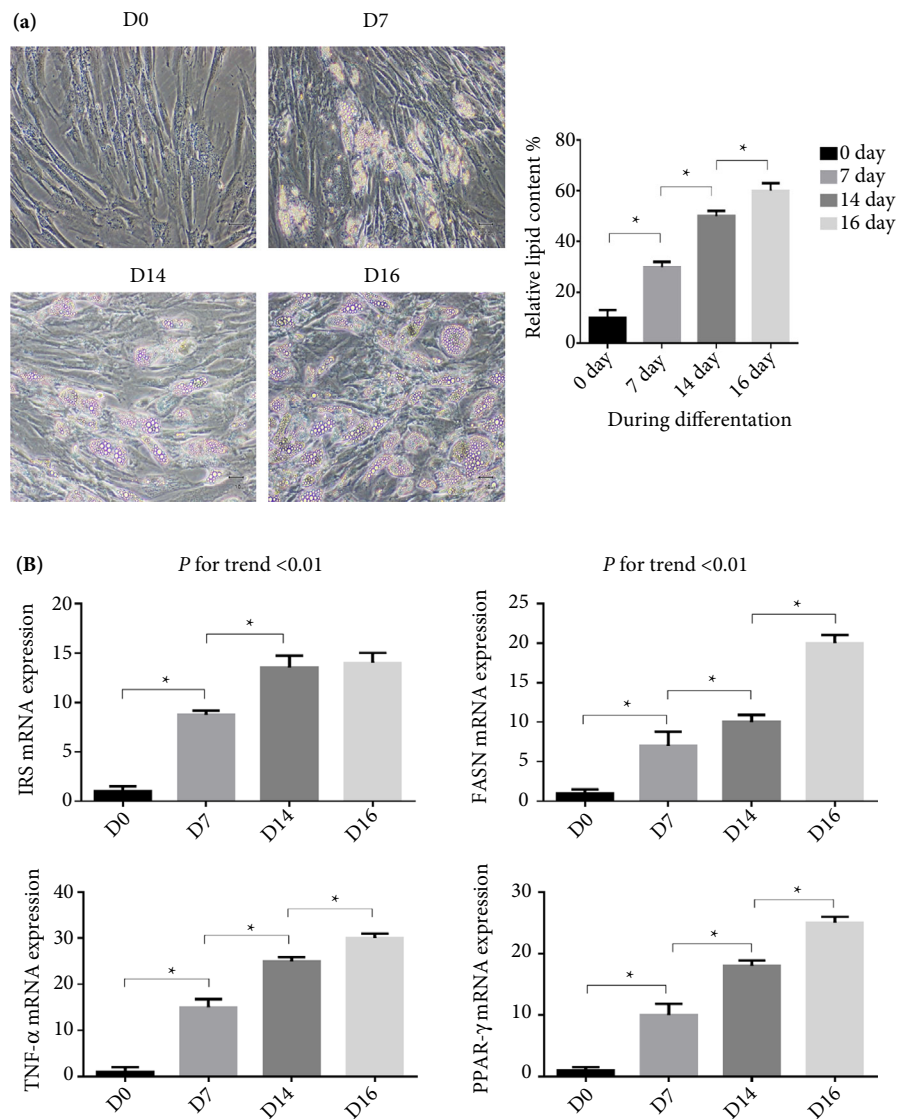


Figure 1 | The morphology and expression of insulin resistance markers during adipocytes differentiation. (a) Preadipocyte Simpson Golabi Behmel Syndrome cells were induced to differentiate for the indicated times, and the morphology of day-0 (D0), day-7 (D7), day-14 (D14) and day-16 (D16) adipocytes (magnification: $\times 10$ and $\times 40$). The quantification of lipid content by Oil Red O staining. (b) Insulin resistance markers (insulin receptor substrate [IRS], fatty acid synthase [FASN], tumor necrosis factor- α [TNF- α], peroxisome proliferator-activated receptor gamma [PPAR- γ]) expression during differentiation of adipocyte by real-time polymerase chain reaction. Data are presented as the mean \pm standard deviation. All P for trend < 0.01 and $*P$ < 0.05. mRNA, messenger ribonucleic acid.

high-fat diet group, the expressions of Gas6 and adiponectin were decreased compared with the normal diet group (Figure 4b). These findings strongly suggested that the expression of Gas6 might be associated with and modulate the expression of adiponectin.

Metformin ameliorated insulin resistance by enhancing the expression of Gas6

To examine how Gas6 influences adiponectin secretion during adipocyte differentiation, we used metformin, a first-line drug

for the treatment of type 2 diabetes mellitus for the experiment, as some studies have shown that metformin can enhance the expression of Gas6 through AMPK activation. After treating TZD day-16 adipocytes with metformin (2 mmol/L) for 6 h, the Gas6 expression was increased (Figure 5a). We then examined adipocytokine gene expressions (adiponectin, leptin, resistin and retinol-binding protein 4), and the results showed that the expression of adiponectin was increased in the TZD only treatment group, and metformin facilitated more expression of adiponectin than the TZD only treatment day-16 adipocytes

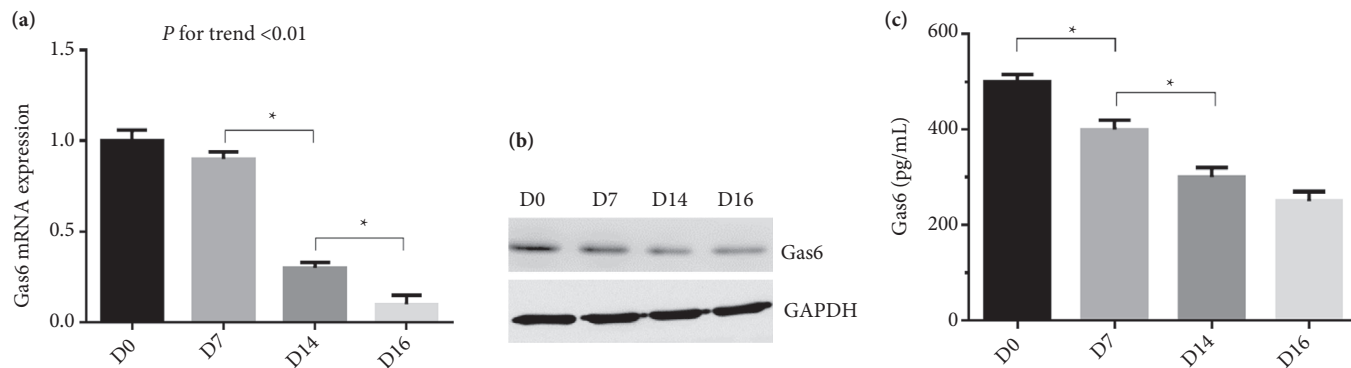


Figure 2 | Growth arrest-specific 6 (Gas6) expression during the differentiation of adipocyte. (a) The messenger (ribonucleic acid) and (b) protein of Gas6 expression during the differentiation of adipocytes by real-time polymerase chain reaction and western blot. (c) The secretion of Gas6 proteins during the differentiation of adipocytes by enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation. All *P* for trend < 0.01 and **P* < 0.05.

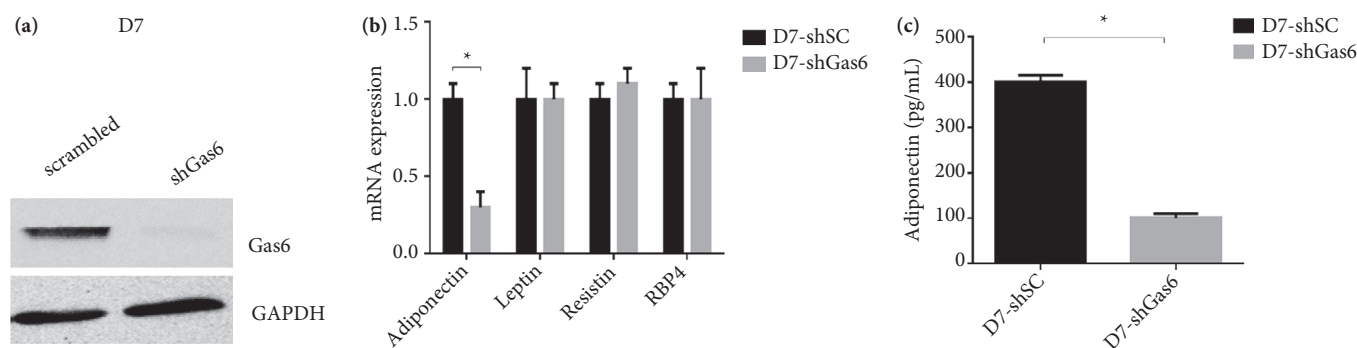


Figure 3 | Growth arrest-specific 6 (Gas6) modulated the expression of adiponectin in adipocytes. (a) The efficiency of Gas6 knockdown in day-7 (D7) adipocytes. (b) The adipocytokines gene expression (leptin, adiponectin, resistin and retinol-binding protein 4 [RBP4]) after knockdown Gas6 in D7 adipocytes by real-time polymerase chain reaction. (c) The adiponectin expression after knockdown Gas6 in D7 adipocytes by enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation. **P* < 0.05. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger ribonucleic acid.

(Figure 5b,c). These results showed that metformin can ameliorate insulin resistance by inducing AMPK and Gas6, which in turn enhances adiponectin secretion during adipocyte differentiation.

DISCUSSION

Obesity increases chronic inflammation and causes obesity-related diseases, such as type 2 diabetes and atherosclerosis¹³. The development of obesity arises from either hypertrophy of individual adipose cells due to lipid accumulation or hyperplasia of adipocytes on differentiation of precursor cells into mature adipocytes, followed by adipocyte inflammation and insulin resistance¹⁴. Gas6, a gamma-carboxyglutamic acid domain-containing protein, was first identified in 1988 by

Schneider *et al.*¹⁵ while screening for genes that had an increased expression in mouse fibroblast cells under growth arrest conditions. The functions of Gas6 include regulation of cell proliferation, differentiation, migration and production of related inflammatory factors. In addition, Gas6 also affects differentiation of immune cells and oil droplet synthesis of adipocytes, indicating that Gas6 is involved in various diseases⁵.

Lijnen *et al.*¹⁴ found that Gas6 was increased in 3T3-F442A preadipocytes, and decreased with the degree of differentiation. It was also found that adipogenic markers were increased, whereas Gas6 was decreased during adipocytes differentiation. Therefore, Gas6 might affect adipogenesis⁶. Furthermore, Gas6 is closely associated with impaired glucose tolerance, endothelial dysfunction and kidney disease, and is even reported to be an

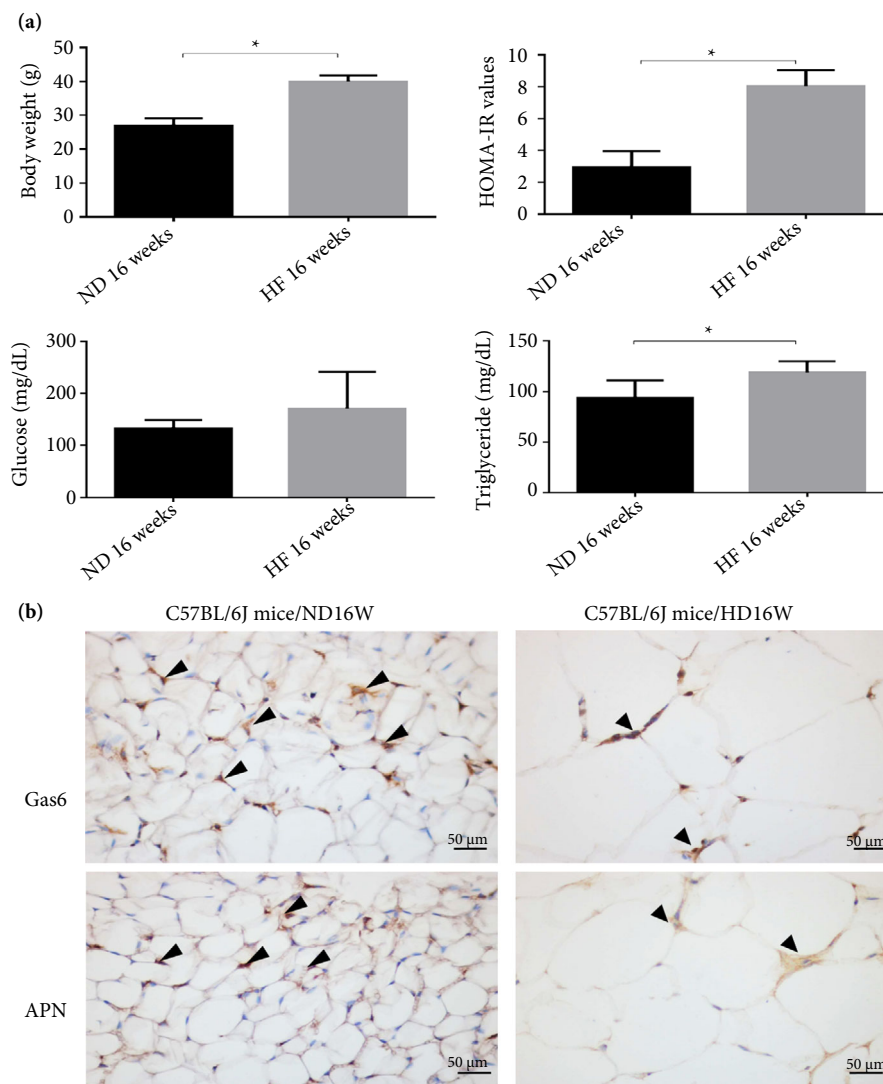


Figure 4 | The expression of growth arrest-specific 6 (Gas6) and adiponectin in insulin-resistant obese mice. Mice fed a normal diet (ND; 10% of calories from fat) or high-fat diet (HF; 60% of calories from fat) for 16 weeks, and blood samples were collected for measurement of blood glucose and insulin. (a) Bodyweight, fasting blood glucose, homeostasis model of assessment of insulin resistance and triglycerides levels are shown. (b) Immunohistochemistry images of Gas6 and adiponectin in adipocyte tissue (magnification: $\times 40$). Data are presented as the mean \pm standard deviation. * $P < 0.05$; $n = 10$ animals per group.

independent risk factor for type 2 diabetes¹⁶. The average amplitude of blood glucose and chronic inflammation in diabetes patients can also lead to the downregulation of Gas6 signaling¹⁷, suggesting that Gas6 might play a potential role in the regulation of insulin resistance. Furthermore, Gas6 has been shown to be involved in the processes of proliferation, differentiation and inflammatory responses in various tissues and cells^{18,19}. Although the role of Gas6 signaling in the inflammation of adipose tissue remains controversial, some studies showed that Axl knockdown in adipocytes led to no significant change in adipogenesis⁴, suggesting that Gas6 might interact with adipocytes by other unknown mechanisms.

Previous clinical studies showed that Gas6 expression was significantly reduced in elderly patients with type 2 diabetes compared with healthy groups, whereas plasma C-reactive protein was significantly increased, suggesting that hyperglycemia and insulin resistance might affect circulating Gas6 levels and inflammation^{16,20}. A recent study showed that fat tissue is an endocrine organ that can produce and secrete a variety of adipocytokines and adipokines^{21,22}. In the present study, we showed that Gas6 was associated with specific cytokine expressions from adipocytes and that it altered insulin resistance. In addition, we found that adiponectin played a crucial role in the Gas6 modulation of insulin resistance. Increasing evidence also

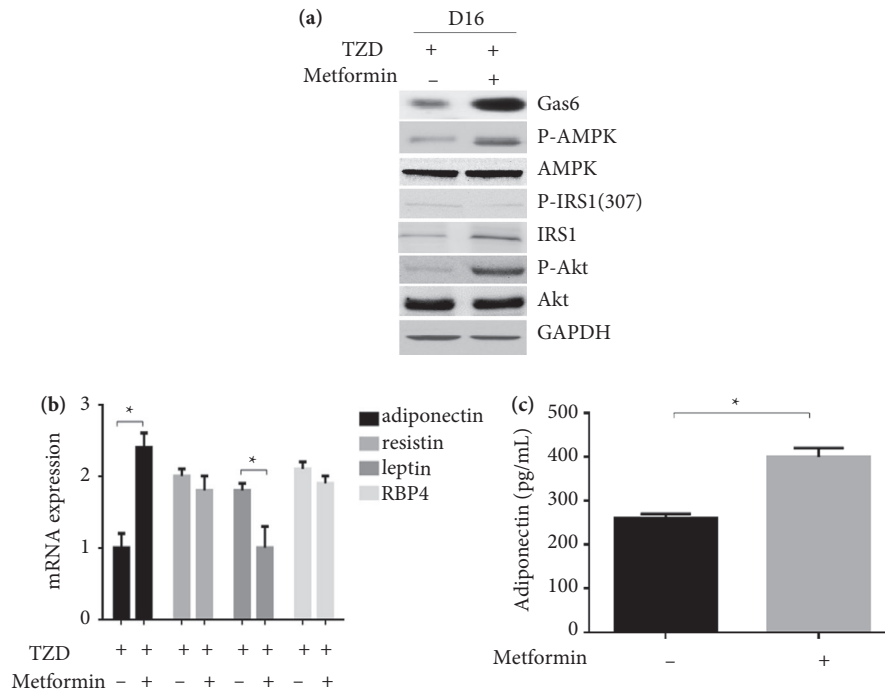


Figure 5 | Metformin ameliorated insulin resistance by enhancing the expression of growth arrest-specific 6 (Gas6). Using thiazolidinedione (TZD) treatment day-16 (D16) adipocytes treated with metformin (2 mmol/L) for 6 h. (a) The protein of Gas6, phospho-adenosine monophosphate-activated protein kinase (p-AMPK), adenosine monophosphate-activated protein kinase (AMPK), phospho-insulin receptor substrate-1 (p-IRS1[307]), insulin receptor substrate-1 (IRS1), phospho-protein kinase B (p-Akt) and protein kinase B (Akt) by western blot. (b) The adipocytokine gene expressions (adiponectin, leptin, resistin and retinol-binding protein 4 [RBP4]) by real-time polymerase chain reaction. (c) The adiponectin expression after metformin treatment in day-16 (D16) adipocyte by enzyme-linked immunosorbent assay. Data were presented as the mean \pm standard deviation. * $P < 0.05$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

indicates that adiponectin modulates the resolution of inflammation²². Furthermore, in the adiponectin knockout mouse model, Takemura *et al.*²³ found that the expression of pro-inflammatory cytokines were increased, whereas the expression of anti-inflammatory cytokines were decreased, indicating that high levels of adiponectin can increase the anti-inflammatory response. This strategy can prevent types of obesity-related diseases²³. The results of the present *in vitro* experiments are also consistent with the data of our animal study, and both Gas6 and adiponectin expressions from the adipocyte tissues of high-fat diet-induced obese mice appeared to be lower compared with the chow-fed group. Taken together, the present findings showed adipocyte differentiation from preadipocytes to mature adipocytes through Gas6 signaling of adipocytes with related effects on adiponectin expression, and that this was more prominent in insulin resistance. These results were also found in the animal model.

Metformin is a first-line drug commonly used to treat diabetes mellitus, and it has been shown to mitigate hyperglycemia through many potential molecular mechanisms, one of which is activation of AMPK. Due to the downregulation of AMPK in insulin resistance and adiposopathy²³, suppressed Gas6 levels might play a role

in AMPK inactivation, particularly in conditions of obesity or insulin resistance. Some recent studies have shown that metformin can enhance Gas6 expression through AMPK activation²⁴. Metformin might increase serum adiponectin levels in type 2 diabetes mellitus patients²⁵, and metformin is able to upregulate adiponectin gene expression in subcutaneous adipocytes²⁶. As shown in the present study, metformin ameliorated insulin resistance through the activation of AMPK and Gas6, which in turn enhanced adiponectin expression. However, there was no experiment, such as knockdown of Gas6 would lead to lesser amelioration of insulin resistance induced by metformin. This part requires more research to confirm. This novel finding might further support the potentially therapeutic role of Gas6 in adiposopathy of obesity and insulin resistance in the future.

In the present study, we found that Gas6 facilitated the expression of adiponectin in adipocytes, which then ameliorated adipose tissue inflammation in conditions of obesity and insulin resistance. However, a limitation of this study was that only one role of Gas6 in the pathogenesis of adiposopathy in obesity was elucidated, and further studies are warranted to investigate the highly complex underlying pathophysiological mechanisms of Gas6 and adipose tissue.

ACKNOWLEDGMENTS

We sincerely thank the study staff for their technical assistance, particularly Miss Wang and Miss Hsu for the laboratory measurements. This work was supported by grants from the Tri-Service General Hospital (TSGH-C105-005-S03, TSGH-C107-006-006-S02, TSGH-C108-005-006-006-S02, TSGH-C107-103, TSGH-C108-143, MAB-107-063, MAB-108-045, MAB-106-113, MAB-108-046) and from the Ministry of Science and Technology (MOST 107-2314-B-016-007, MOST 105-2314-B-016-040-MY3, MOST 108-2314-B-016-033-MY2, MOST 108-2314-B-016-019-MY3, MOST 108-2314-B-016-019-MY3, MOST 108-2314-B-016-013).

DISCLOSURE

The authors declare no conflict of interest.

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