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SCHOOL OF MEDICINE

Thesis

REGULATION OF ATGL-MEDIATED LIPOLYSIS BY FSP27/CIDEC IN HUMAN ADIPOCYTES

by

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B.Sc., National University of Singapore, 2006

Submitted in partial fulfillment of the

requirements for the degree of

Master of Arts

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REGULATION OF ATGL-MEDIATED LIPOLYSIS BY FSP27/CIDEC IN HUMAN ADIPOCYTES

RAJWEEN KAUR

Boston University School of Medicine, 2013

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ABSTRACT

Increased free fatty acid (FFA) flux from adipocytes due to increased lipolysis, has a key contribution in the pathophysiology of metabolic disease. There is a lack of knowledge of the molecular components which determine the TG storing capacity and lipolysis in adipocytes. Studies from our lab and others have demonstrated the role of a lipid droplet associated protein, Fat Specific Protein 27 (FSP27, also called CIDEC), in regulating triglyceride accumulation and lipolysis in adipocytes, but its mechanism of action remains elusive. In the present study, we used cultured human primary adipocytes to define the role of FSP27 in regulating both basal and isoproterenol-stimulated lipolysis. Using a combination of RNAi and adenoviral mediated overexpression techniques, we have shown that FSP27 regulates ATGL-mediated lipolysis by down-regulating gene and protein expression of ATGL. Furthermore, our data shows that FSP27-mediated triglyceride accumulation is suppressed in the absence of ATGL. Our

results support a model whereby FSP27 regulates ATGL-mediated lipolysis to accumulate triglycerides in human adipocytes.

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ABBREVIATIONS

α-MEM	α-Modified Eagle's Medium
ATGL	Adipose Triglyceride Lipase
BSA	Bovine Serum Albumin
cAMP	cyclic-AMP (Adenosine Monophosphate)
cDNA	Complimentary Deoxyribonucleic Acid
CE	Cholesterol Ester
CFP	Cyan Fluorescent Protein
CGI-58	Comparative Gene Identification-58
CIDEA	Cell Death-Inducing DNA Fragmentation Factor-Like Effector A
CIDEB	Cell Death-Inducing DNA Fragmentation Factor-Like Effector B
CIDEC	Cell Death-Inducing DNA Fragmentation Factor-Like Effector C
DAG	Diacylglycerol
ddH_2O	Double-Distilled Water
DMEM	Dulbecco's Modified Eagle's Medium
FA	Fatty Acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFA	Free Fatty Acid
Fig.	Figure
FSP27	Fat-Specific Protein of 27kDa
g	Gram

G0S2	G0/G1 Switch Gene 2
HA	Human Influenza Hemagglutinin
Hrs	Hours
HSL	Hormone Sensitive Lipase
IBMX	3-isobutyl-1-methylxanthine
kDa	Kilodalton
KRB	Krebs-Ringer Buffer
LD	Lipid Droplet
MAG	Monoacylglycerol
MGL	Monoacylglycerol Lipase
ml	Milliliter
m.o.i	Multiplicity of Infection
mRNA	Messenger RNA
NEFA	Non-Esterified Fatty Acid
PKA	Protein Kinase A
PLIN1	Perilipin 1
PVDF	Polyvinylidene Difluoride
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
siRNA	Small Interfering RNA
siScr	Small Interfering RNA-Scrambled

TAG	Triacylglyceride
TBS-T	Tris-Buffered Saline Tween 20
TG	Triglyceride
Tris-HCl	Tris(hydroxymethyl)aminomethane Hydrochloride
T3	Triiodo-l-thyronine

INTRODUCTION

The adipose tissue functions as the primary fuel depot in vertebrates, and its ability to store energy as triacylglycerols (TAGs) in intracellular lipid droplets (LDs) and, on demand, to mobilize these stores via lipolysis, is a highly conserved process that is essential for survival (1). Lipid droplets (LDs) are intracellular organelles that are composed of a neutral core of triglycerides (TG) and cholesterol esters (CE) that are enclosed by a monolayer of phospholipids (2,3). Lipolysis is a catabolic branch of the fatty acid (FA) cycle that provides FAs in times of metabolic need. FAs are essential substrates for energy production and the synthesis of most lipids. Despite their fundamental physiological importance, an oversupply of FAs is highly detrimental as increased free fatty acids (FFA) causes lipotoxicity which can cause secondary metabolic complications such as insulin resistance (4).

The rate of adipocyte lipolysis in many obese individuals is constitutively increased, resulting in elevated levels of circulating FFAs, which may be stored as TAG in LDs within skeletal muscle and liver (4). Adipocytes are able to secrete FFAs and provide them as systemic energy substrate, whereas non-adipose cells do not secrete FFAs but utilize them in a cell autonomous manner for energy production or lipid synthesis (5). Both local and circulating FFAs are important etiologic agents in the development of insulin resistance, hyperlipidemia, inflammation, and hepatic steatosis (4). Thus, the balance

between TG accumulation and storage, and hydrolysis via lipolysis, called TG turnover, is a very important mechanism that needs to be well regulated.

According to the current model of lipolysis, the hydrolysis of TGs to FAs and glycerol requires three consecutive steps that involve three different enzymes- adipose tissue glycerol lipase (ATGL; also called desnutrin or PNPLA2), Hormone sensitive lipase (HSL), and Monoacylglycerol lipase (MGL). ATGL is highly expressed in adipose tissue and is the rate-determining enzyme which first initiates lipolysis by specifically removing the first fatty acid from TAG to produce DAG substrate, which is then hydrolyzed by HSL to generate an additional fatty acid and MAG substrate. MAGs are converted into fatty acid and glycerol by MGL in the final step of lipolysis (6). Although HSL is mainly regulated by protein kinase A (PKA) phosphorylation, ATGL is coactivated by the protein comparative gene identification-58 (CGI-58) and inhibited by the protein G0/G1 switch gene 2 (1).

Under basal conditions in adipocytes, Perilipin A (PLIN1) prevents the access of CGI-58 to ATGL, thus decreasing lipolysis (7,8,9). ATGL is mostly cytoplasmic under these basal conditions, where a small fraction of ATGL is in complex with its inhibitor, G0/G1 switch gene 2, G0S2 (6). Upon β-adrenergic stimulation, PKA activation results in phosphorylation of PLIN1 at multiple sites, causing the release of CGI-58. CGI-58 then binds and stimulates ATGL (7,10). ATGL then translocates from the cytoplasm to the surface of the LDs and together with HSL hydrolyse TG. Prolonged β-adrenergic stimulation

subsequently downregulates protein level of G0S2, thereby releasing more ATGL for sustained lipolysis.

The CIDE family proteins CIDEA, CIDEB and FSP27/CIDEC are also LD-associated proteins and are closely linked to the development of metabolic disorders, including obesity, diabetes and liver steatosis (11). FSP27 has been shown to be crucial for the fusion of smaller LDs into larger ones (12,13) triglyceride (TG) accumulation (14,15,16,17,18). Higher expression of CIDEC was found in visceral fat of insulin sensitive compared to insulin resistant obese humans (19). Also, a study showed that a mutation in CIDEC results in multilocular adipocytes associated with partial lipodystrophy and insulin resistance in a human subject (20). A recent study from our lab has also shown that FSP27 plays an important role in regulating LD morphology, whereby it interacts with PLIN1 in human adipocytes to promote the formation of large lipid droplets (21).

OBJECTIVES OF THE THESIS

The main objective of this study was to define the role of FSP27 in regulating ATGL-mediated lipolysis in human adipocytes. Expressing FSP27 in various cell types markedly enhances TG deposition and LD size, whereas FSP27 depletion both *in vitro* and *in vivo* causes fragmentation of LDs and increases TG hydrolysis in adipocytes (12,14,15,17). Another recent study has shown that PLIN-1 and FSP27 work in concert to control lipolysis in 3T3-L1

adipocytes (1).

Recent discoveries have highlighted the role of various proteins in the lipolytic machinery, however the complexity of lipolysis and its regulation is still not completely understood. In particular, the role of FSP27 in regulating lipolysis, and its interaction with ATGL has not been studied in human adipocytes. Given that FSP27 has been shown to be associated with LDs, we hypothesized that FSP27 might be regulating lipolysis to decrease production and circulation of FFAs. Thus, peripheral organs (such as skeletal muscle and liver) are protected from increased FFA uptake and secondary metabolic complications such as insulin resistance. In this study, we used cultured human adipocytes as a model system to test our hypothesis.

Primary pre-adipocytes were harvested from white adipose tissue of human subjects. These primary adipocytes were then cultured and differentiated. In line with studies done in 3T3-L1 adipocytes by Puri et al (15), we had to first establish successful and sustainable knockdown in these cultured human adipocytes. Following this, we measured total basal and stimulated (using isoproterenol, a β-adrenergic agonist) lipolysis in the FSP27-depleted adipocytes. FSP27 depletion resulted in increased basal and stimulated lipolysis in the human adipocytes. In contrast, over-expression of FSP27 results in decreased basal and stimulated lipolysis, as compared to control cells. These results suggest that FSP27 regulates ATGL-mediated lipolysis. To further test this hypothesis, we depleted ATGL in the cells and over-expressed FSP27 to see if

there is a further reduction of lipolysis in the absence of ATGL. Indeed, we found that in the absence of ATGL, there is no further decrease in lipolysis, which suggests that FSP27 interacts with ATGL to decrease lipolysis and accumulate TGs in human adipocytes.

MATERIALS AND METHODS

Cell Culture

Human pre-adipocytes were procured from the Boston Nutrition Obesity Research Center adipocyte core. Cells were seeded at a density of 5,000 cells/ml/well in a 12-well plate, in Growth Medium which was made using 13.5g of α-MEM powder (Gibco) with 10% FBS (Gibco), 100units/ml of penicillin and streptomycin (Gibco) and 25mM Sodium Bicarbonate (Fisher Scientific) reconstituted in double-distilled water (ddH_2O) to 1 liter, pH 7.2-7.3. This growth medium was changed every 2 days, until cells reached 90% confluence. In order to allow the cells to differentiate, the GM was then replaced by Complete Differentiation Medium, which consisted of: 13.5g of Dulbecco's Modified Eagle's medium powder (DMEM)/F12 (Gibco), 100units/ml of penicillin and streptomycin (Gibco), 15mM Hepes (Sigma), 25mM Sodium Bicarbonate (Fisher Scientific), 33μM Biotin (Sigma), 17μM Pantothenate (Sigma), 0.5mM IBMX (Sigma), 10mg/L Transferrin (Sigma), 1μM Rosiglitazone (BioMol), 100nM Dexamethasone (Sigma), 100nM Human Insulin (Recombinant), 2nM triiodo-l-thyronine (T3) (Sigma), reconstituted in ddH_2O to 1liter, pH 7.4. The day cells were first given Complete Differentiation Media is designated as Day 0. The media was changed every 2 days until the pre-adipocytes were fully differentiated into mature human white adipocytes that were filled with lipid droplets. Following differentiation, the cells were maintained in Maintenance

medium until fully differentiated with the media being changed every 2 days. Maintenance medium was made of the similar constituents as Complete Differentiation media, except without Rosiglitazone, IBMX, T3 and Transferrin. Additionally, the concentrations of Human Insulin and Dexamethasone used was 10nm each.

siRNA Treatment and Adenoviral Transduction

The following siRNA constructs were used for experiments: siScr (from Qiagen)- All Stars Negative Control; human siFSP27 (from Dharmacon)- AAC-UGU-ACA-GAC-AGA-AGA-GUA-UU; siATGL (from Invitrogen)- ACC-UGC-CAC-UCU-AUG-AGC-UUA-AGA-A. On Day 9 following differentiation, cultured adipocytes were transfected with 40nm siRNA, 5µl/well PLUS reagent and 3µl/well Lipofectamine reagent all from Invitrogen, in a total volume of 350µl/well (100µl siRNA mixture, 250µl MM). 24hrs post-transfection, 500µl Maintenance Media was added to cells. The media was then changed 24hrs later with 1ml of Maintenance Media. Cells were further incubated for 3 days (Day 14) before being harvested for analysis. FSP27-HA tagged adenoviruses were generated at the Adenoviral Vector Core Facility at Tufts Medical Center. ATGL-CFP and FSP-CFP adenovirus was a kind gift from Dr Carol Sztalryd and Dr Dawie Gong from the University of Maryland. The adenovirus was added to adipocytes on either Day 9 (if experiment was solely studying effects of over-expression) or on Day 14 (for

re-expression analysis) of differentiation at m.o.i of 100. Cells were then harvested 48hrs post-infection for analysis.

RNA Isolation and qPCR

Total RNA was isolated from human adipocytes from each well (12-well plate) with 1ml TRIzol (Invitrogen). Total RNA isolation was performed as described in the manufacturer's protocol and RNA concentrations were measured on a Nanodrop^{TM} nd-1000 spectrophotometer (Thermo-Scientific). For quantitative PCR (mRNA analysis), 1 µg of RNA was reverse transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche). The cDNA was diluted 1:10 and 1µl was used for each reaction (per well, 384-well plate) in triplicates. Primers (TaqMan) were used with Roche LightCycler 480 Probes Master kit (Roche Applied Science) at 0.25µl, with 2.5µl Master stock and 1.25µl ddH_2O per reaction. Thermal cycling conditions were: 10 min at 95°C for activation, 45 cycles of 10 seconds at 95°C, 30 seconds at 60°C and 1 second at 72°C for reactions, and 5 min at 37°C for cooling down. Comparative-ΔCt method was used to calculate relative changes in mRNA abundance.

Protein Quantification and Analysis

Cells were lysed in Cell Lysis Buffer (Cell Signaling) with 5% SDS, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). Protein lysates were then briefly sonicated, incubated in a 37°C water bath for 30

minutes, and then centrifuged at 10,000rpm for 10 minutes. The clear middle phase of the solution was then collected as the protein sample. Protein quantification was performed using the Pierce BSA Protein Assay Kit (Thermo Scientific). Immunoblots (Western Blot) were performed using 10% and 15% Tris-HCl gels (Bio-Rad), run at 100 volts for 90 minutes, and transferred to PVDF membranes (Bio-Rad), run at 60 volts, for 120 minutes. Primary antibodies used were all diluted in 2% BSA in TBS-T. Antibodies used were: mouse anti-β-tubulin (Invitrogen, 1:5000 dilution), rabbit anti-FSP27 (1:4000 dilution), and rabbit IgG anti-ATGL (1:5000 dilution). Secondary antibodies were purchased from Santa Cruz Biotechnology (1:10000 dilution).

Triglyceride Accumulation and Lipolysis

For analysis of total cellular glycerol release (measure of lipolysis), cells were incubated in Krebs-Ringer buffer (KRB) with 4% BSA for 2.5 hours. At the end of the 2.5 hours, the KRB was collected and analyzed for glycerol release (basal lipolysis). For stimulated lipolysis, after 1.5 hours of incubation with KRB, cells were treated with 1μm Isoproterenol (Sigma), a β-adrenergic agonist, for 1 hour. For total cellular triglyceride (TG) content analysis, after collection of the KRB buffer supernatant, cells were harvested with 1x SDS Triglyceride lysis buffer (0.1% SDS, 1mM EDTA and 100mM Tris-HCl, adjusted to pH 7.4). Total cellular triglyceride content and basal/stimulated lipolysis assays were measured by using the Serum Triglyceride Determination Kit from Sigma-Aldrich according to

the manufacturer's instructions. Concentrations were determined by normalization to the standard curves; TG was further normalized to total protein content, and lipolysis was normalized to TG content and molecular weight of glycerol (92.09 g/mol).

Statistics

Quantitative data are presented as mean ± standard error. Statistical significance was calculated using paired student's t-test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Knockdown of FSP27 using siRNA is stable for at least up to 10 days after siRNA transfection.

The main purpose of this preliminary experiment was to establish that the siRNA construct for FSP27 works and that we were able to achieve significant and sustainable knockdown of FSP27 in human adipocytes. Human white adipocytes were differentiated as described in methods. On day 9 of differentiation, the cells were treated with 40nm siScr (control) or siFSP27 for 5 or 10 days. Following these time points, the cells were harvested and the protein expression levels of FSP27 and endogenous control, β-tubulin, were measured by Western Blotting. As shown in Fig. 1, we were able to achieve greater than 90% knockdown of FSP27 in human adipocytes after 5 days of transfection. This knockdown was

sustainable even after 10 days post-transfection, which shows about 90% knockdown in FSP27 protein expression compared to the negative control, siScr. We thus established that 40nm of siRNA was sufficient to induce knockdown of FSP27 in cultured human adipocytes in 5 days.

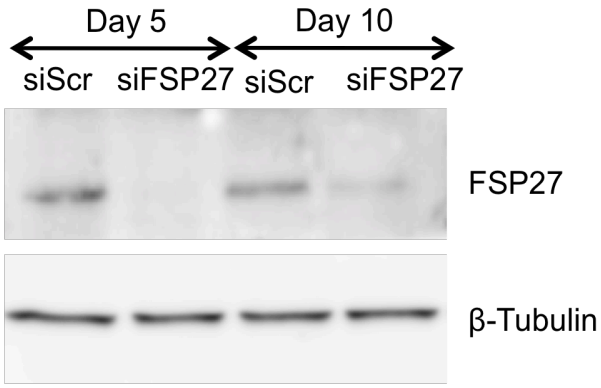

Fig. 1 **Protein expression of FSP27 and β-tubulin in control siSCR and siFSP27 treated cells at 5 and 10 days post-siRNA treatment.** Primary cultures of mature white adipocytes were grown, differentiated and treated with siRNA as described in methods. siSCR (scrambled) was used as a control. Protein lysates were loaded at 15ug/lane and probed with antibodies against FSP27 and β-tubulin. Image is representative of at least three independent experiments.

FSP27 depletion increases both basal and stimulated lipolysis in human adipocytes.

Studies from our lab and others have shown the role of FSP27 in lipid droplet dynamics and lipolysis in 3T3-L1 adipocytes (1,15). In order to investigate if FSP27 plays a role in both basal and stimulated lipolysis in human adipocytes, we used siRNA-mediated approach to knockdown FSP27 in cultured human adipocytes, as established from Fig. 1. In order to investigate the role of FSP27 depletion on basal and stimulated lipolysis, we measured free glycerol release

(as a measure of lipolysis) in cells at basal level (no treatment) and upon stimulation with a β-adrenergic agonist, isoproterenol (1μm, 1 hour). As shown in Fig. 2, FSP27 depletion caused a 20% increase in basal lipolysis and a 40% increase in stimulated lipolysis. These results suggest that FSP27 plays a role in both basal and stimulated lipolysis in human adipocytes.

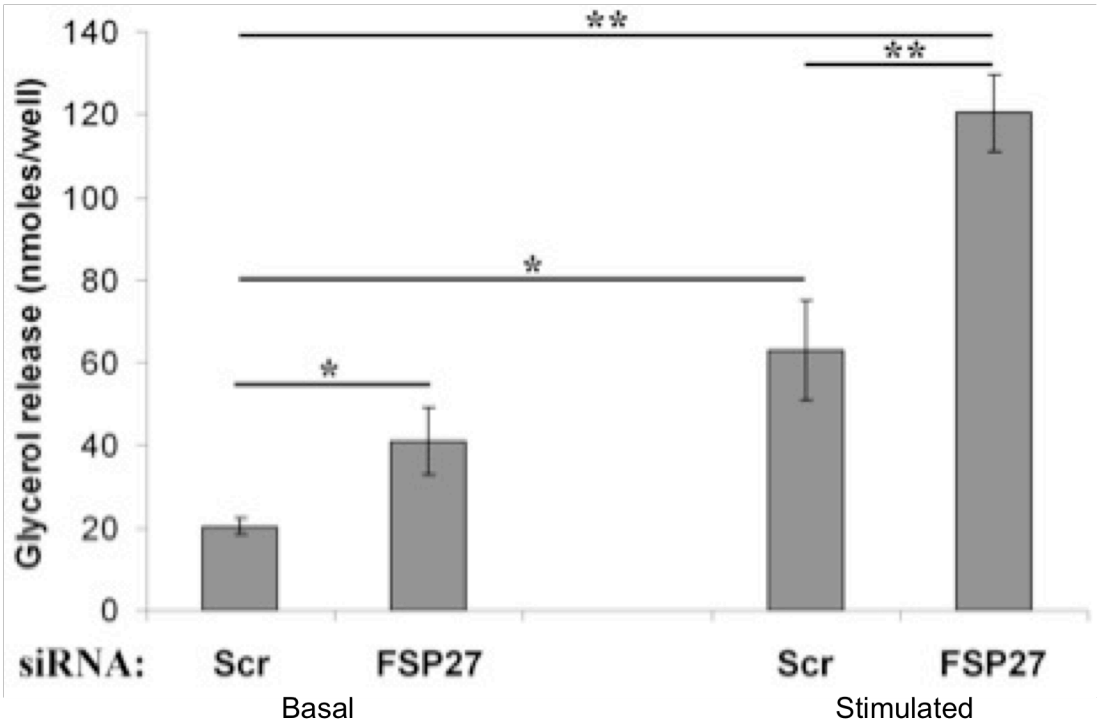

Fig. 2 **Basal and stimulated lipolysis in siSCR and siFSP27 treated cells.** Primary adipocytes were cultured, differentiated and treated with siRNA as described in methods. 5 days post-siRNA treatment, cells were treated with KRB albumin buffer (basal) or with KRB albumin buffer with β-agonist isoproterenol (stimulated), and harvested for total glycerol release as described in methods. The data show an average of three independent experiments. Values are means ± standard error; $^{*}p < 0.05$ (paired *t*-test).

FSP27 decreases ATGL-mediated lipolysis in human adipocytes.

ATGL is a hydrolase that controls the rate-determining step in lipolysis and is critical for regulating lipolysis in human adipocytes. Based on our results in Fig. 2 showing that depletion of FSP27 promotes both basal and stimulated lipolysis, we hypothesized that FSP27 might regulate ATGL-mediated lipolysis. To investigate the role of FSP27 in regulating ATGL activity we first performed co-immunoprecipitation to analyze if FSP27 interacts with ATGL. This experiment was performed by Dr Grahn in our lab, and she found that indeed, FSP27 interacts with ATGL *in vitro* (manuscript in preparation). Cultured adipocytes were infected with adenoviral preparations of FSP27-HA, ATGL-CFP, or both, as described in the methods.

As expected in Fig. 3, over-expression of FSP27 caused a 3.5 fold decrease in basal lipolysis ($p<0.05$) and a 30% decrease in stimulated lipolysis, as compared to the control. In contrast, over-expression of ATGL caused a 60% increase in basal lipolysis, and an 82% increase in stimulated lipolysis when compared to control. However, when expressed in conjunction with FSP27-HA, ATGL-mediated increase in basal and stimulated lipolysis was suppressed by 52% and 70% respectively, when compared to ATGL on its own. It is interesting and important to note that we did not see as much as an increase in stimulated lipolysis in the control cells as compared to the huge induction we saw in Fig. 2. This is because there is usually some residual side-effects of treatment with adenovirus and the control cells were treated with adenoviral preparation of

eGFP. However, on a whole, these results indicate that FSP27 overrides ATGL-catalyzed lipolysis in human adipocytes.

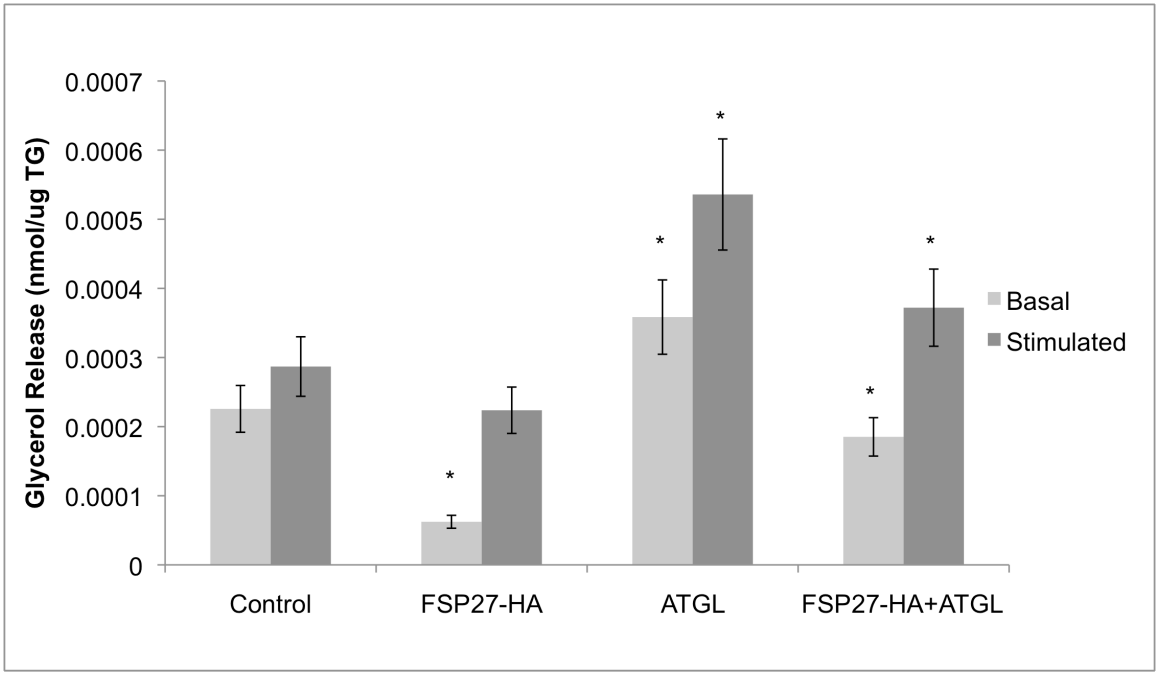

Fig. 3 **Basal and stimulated lipolysis in FSP27-HA and ATGL-CFP treated cells.** Primary adipocytes were cultured, differentiated and treated with adenoviral preparations of FSP27-HA and ATGL-CFP as described in methods. 48hrs post infection, cells were treated with KRB albumin buffer (basal) or with KRB albumin buffer with β-agonist isoproterenol (stimulated), and harvested for total glycerol release as described in methods. Light grey columns represents basal lipolysis, and dark grey columns represent stimulated lipolysis. The data show an average of three independent experiments. Values are means ± standard error; $^{*}p < 0.05$ (paired *t*-test).

FSP27 regulates ATGL gene and protein expression levels.

Given that FSP27 depletion or expression increases or decreases ATGL-mediated basal and stimulated lipolysis respectively, we next examined if FSP27 has any role in regulating ATGL expression. Cultured adipocytes were treated with siRNA against FSP27. Scrambled siRNA was used as a control. 5 days post-transfection, the cells were harvested and RNA was extracted and relative

mRNA expression of FSP27, ATGL and PLIN1 was measured in these cells. FSP27 depletion has previously been shown to increase PLIN1 expression (22). PLIN1 is an adipocyte-specific lipid-droplet associated protein that has been shown to interact with FSP27 to increase FSP27-mediated lipid exchange, lipid transfer and lipid droplet growth (11,21). Functional cooperation between PLIN1 and FSP27 is required for efficient lipid droplet growth in adipocytes, as depletion of either protein impairs lipid droplet growth (11).

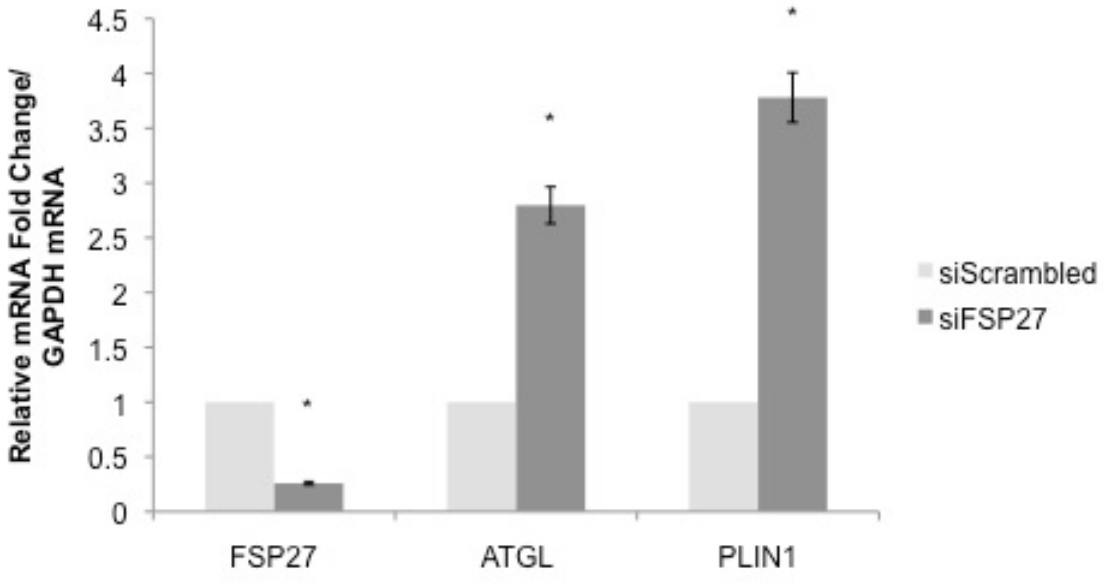

Fig. 4 **FSP27 depletion increases ATGL and PLIN1 gene expression in human adipocytes.** Primary adipocytes were cultured, differentiated and treated with siRNA as described in methods. 5 days post-transfection, RNA was extracted from the cells and mRNA levels were measured by quantitative-PCR. Columns represent relative fold-change in mRNA levels. Light grey columns are cells treated with control siSCR and dark grey columns are cells treated with siFSP27. The data show an average of three independent experiments. Values are means ± standard error; $^{*}p < 0.05$ (paired *t*-test).

As shown in Fig. 4, treatment with siFSP27 resulted in 70% knockdown of FSP27. This decrease in FSP27 mRNA level resulted in a 2.8 fold increase in

ATGL and 3.8 fold increase in PLIN1 mRNA levels. Next, we examined the effect of FSP27 depletion on ATGL protein expression. As shown in Fig. 5, 80% knockdown of FSP27 resulted in 84% increase in ATGL protein expression. This data is consistent with the decrease in the level of genes.

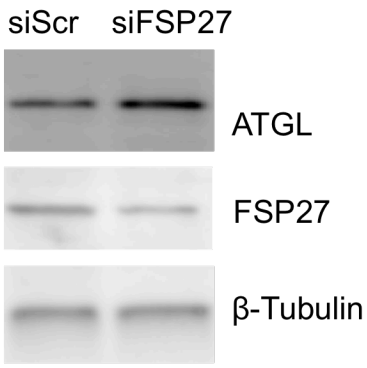

Fig. 5 **FSP27 depletion increases ATGL protein expression in human adipocytes.** Primary cultures of mature white adipocytes were grown, differentiated and treated with siRNA as described in methods. siScr (scrambled) was used as a control. Protein lysates were loaded at 15ug/lane and probed with antibodies against FSP27, ATGL or β-tubulin. Image is representative of at least three independent experiments.

Since FSP27 depletion increases both gene and protein expression of ATGL, this suggests that FSP27 plays a role in regulating its transcription. Therefore, we next wanted to examine if over-expressing FSP27 decreases ATGL gene and protein expression to reduce its hydrolase activity. Cultured adipocytes were treated with either adenoviral preparations of FSP27-CFP, FSP27-HA or siATGL and were then harvested and the RNA was extracted as described in methods. Relative mRNA levels of FSP27, PLIN1 and ATGL were then measured using quantitative PCR. As shown in Fig. 6, infecting cells with FSP27-CFP adenovirus caused 50% increase in mRNA level of FSP27. Also, FSP27-HA adenovirus increased FSP27 expression by 5-fold ($p<0.05$) in human

adipocytes. This correlated with a 90% and 63% increase, respectively, in FSP27 protein levels (Fig.7). However, it is important to note that our qPCR primers detect endogenous FSP27, so somehow infection with the HA-adenovirus increases endogenous FSP27 gene expression.

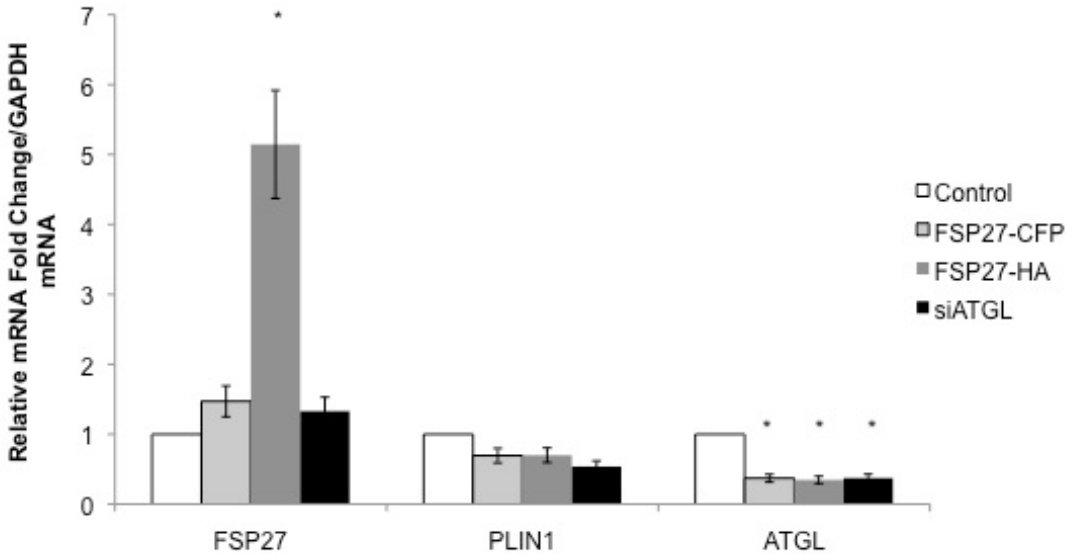

Fig. 6 **FSP27 over-expression decreases ATGL gene expression in human adipocytes.** Cultured adipocytes were treated with adenoviral preparations of FSP-CFP, FSP-HA or siATGL. RNA was extracted from the cells and mRNA levels were measured by quantitative PCR. Columns represent relative fold-change in mRNA levels as compared to endogenous control, GAPDH. The data show an average of three independent experiments. Values are means ± standard error; $^{*}p < 0.05$ (paired *t*-test).

While treatment with siRNA against ATGL resulted in a 70% decrease in ATGL mRNA levels, there seemed to be only a slight increase (30%) in CIDEC mRNA levels. However, cells treated with both FSP27-CFP and FSP27-HA resulted in 63% and 66% decrease in ATGL mRNA levels respectively. This correlates with a 30% and 70% decrease in ATGL protein levels respectively

(Fig. 7). While FSP27 depletion caused an increase in PLIN1 mRNA levels, over-expression of FSP27 resulted in a mild decrease in PLIN1 mRNA levels (about 30%) and a 47% decrease when treated with siATGL. Thus, in combination with the results from Fig. 4 and 5, we can surmise that changes in FSP27 expression modulates ATGL gene and protein expression levels to regulate its hydrolase activity.

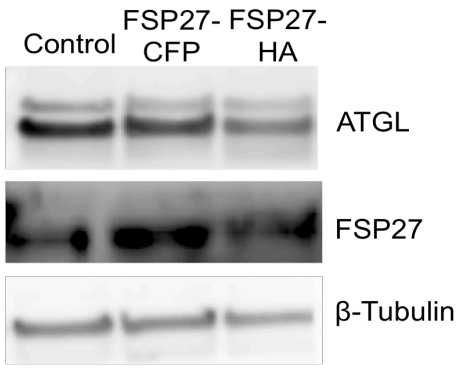

Fig. 7 **FSP27 over-expression decreases ATGL protein expression in human adipocytes.** Cultured adipocytes were treated with adenoviral preparations of FSP27-CFP or FSP27-HA for 48hrs. Cells were then harvested and the protein lysates were loaded at 15ug/lane and probed with antibodies against FSP27, ATGL and β-tubulin. Image is representative of experiment performed in triplicates.

FSP27-mediated TG accumulation is ATGL dependent.

Accumulating evidence supports the role of FSP27 in TG accumulation (14,15). The results thus far have established that FSP27 interacts with ATGL and negatively regulates its activity. As shown in Fig. 8a, in the absence of ATGL (knockdown via siRNA), basal lipolysis is suppressed by 91%. Additionally, as shown before in Fig. 3, exogenous expression of FSP27-HA suppresses lipolysis by 83%. There is no further decrease in lipolysis when cells were depleted of

ATGL and treated with exogenous FSP27. This shows that indeed FSP27 interacts with ATGL to regulate its hydrolytic activity.

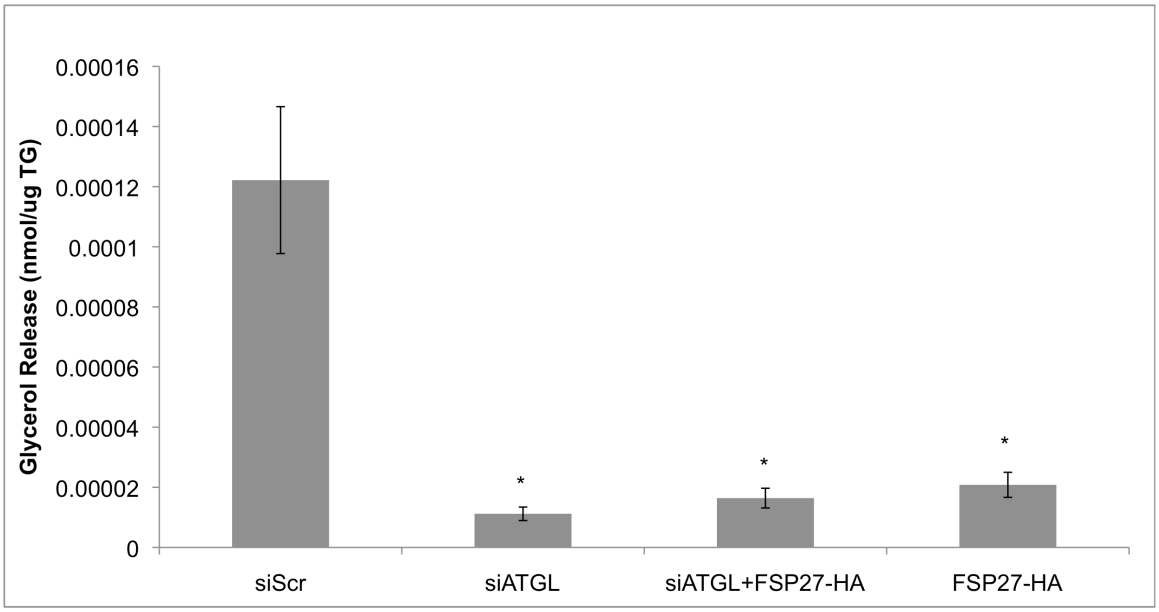

Fig. 8a **FSP27 does not further decrease lipolysis in the absence of ATGL.** Biochemical quantification of total basal lipolysis in Scrambled siRNA (siSCR) and ATGL siRNA (siATGL) treated human adipocytes. 5 days post-transfection, cells were treated with FSP27-HA adenovirus for 48hrs as described in methods. For lipolysis, glycerol released in 2.5hrs was measured and normalized to total celluar triglyceride. The data show an average of three independent experiments. Values are means ± standard error; $^{*}p < 0.05$ (paired *t*-test).

We next hypothesized that FSP27 mediates TG accumulation by suppressing the activity of ATGL. To address this, ATGL was first depleted from cultured human adipocytes using siRNA. FSP27 was then delivered to the adipocytes using adenovirus. The cells were infected with FSP27-HA adenovirus for 48hrs. As shown in Fig. 8b, cells expressing FSP27-HA accumulated significantly higher amount of TG compared to Scrambled siRNA control cells (3-fold increase). However, knockdown of ATGL attenuated the ability of exogenous

FSP27 to accumulate TG, to a level similar to that in cells depleted of ATGL (Fig. 8, second column, siATGL), which is about a 90% increase in TG. This data strongly suggests that FSP27-mediates TG accumulation by inhibiting ATGL-mediated lipolysis (and thus its hydrolase activity), highlighting the role of ATGL-mediated lipolysis in FSP27-mediated TG accumulation.

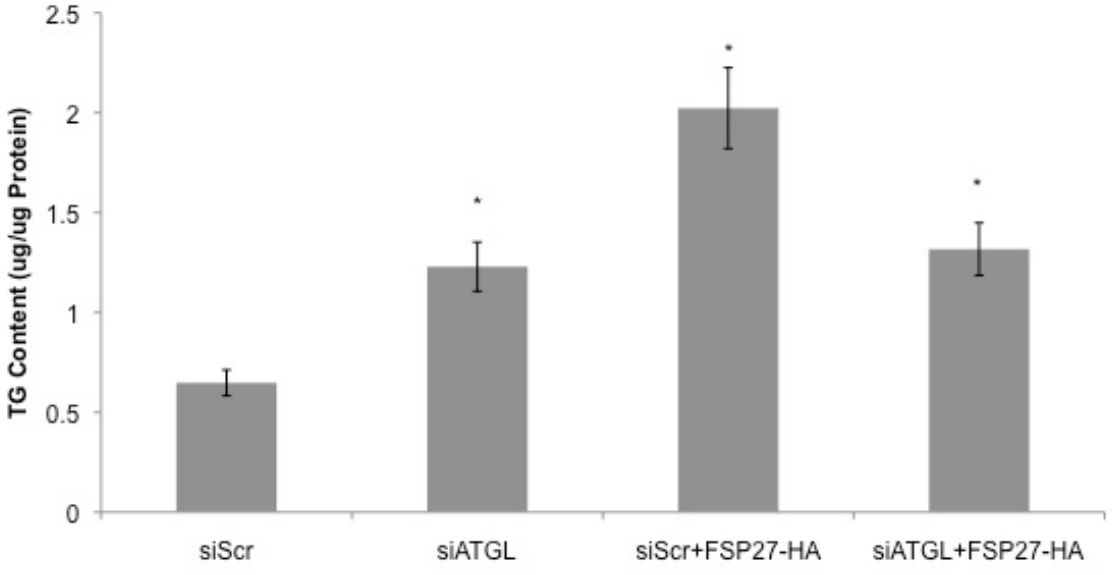

Fig. 8b **FSP27-mediated TG accumulation is ATGL dependent.** Biochemical quantification of total triglyceride in human adipocytes treated with siSCR control and siATGL as described in methods. 5-days post siRNA transfection, cells were treated FSP27-HA adenovirus for 48hrs. Total triglyceride was measured and normalized to total protein. The data show an average of three independent experiments. Values are means ± standard error, $^{*}p < 0.05$ (paired *t*-test).

DISCUSSION

Impaired TG storage and chronic exposure to FFA release from adipocytes via increased lipolysis leads to whole body insulin resistance. This study demonstrates the interaction of a lipid droplet protein, FSP27 with a lipase, ATGL, to regulate lipolysis and improve the lipid storage capacity of adipocytes. The results show that FSP27 interacts with ATGL and reduces its lipolytic capacity. Interestingly, FSP27-mediated TG accumulation was suppressed in the absence of ATGL indicating an important contribution of ATGL-mediated lipolysis in the TG storage capacity of adipocytes. FSP27 depletion increased both basal and isoproterenol stimulated lipolysis in human adipocytes. Importantly, another study in our lab has found that overexpression of FSP27 protected human adipocytes from FFA-mediated insulin resistance (data not shown).

Previous studies showed that depletion of FSP27 increases basal and stimulated lipolysis (1,15) in 3T3-L1 adipocytes. A recent study proposed that FSP27 might limit the LD presence of ATGL due to FSP27's function in regulating LD morphology (1). The absence of FSP27 resulted in multilocularization of LDs, thus it was proposed that increasing total surface area of the LDs due to fragmentation increase the access of ATGL to the LD surface, leading to increased lipolysis. As a matter of fact, a previous study (15) showed that FSP27 depletion resulted in fragmentation of LDs in adipocytes. Interestingly, our recent study (21) showed that overexpression of FSP27 and PLIN1 leads to the formation of enlarged LDs without affecting the lipolytic rates

in cultured human adipocytes. In this study there was no effect of lipid droplet morphology on lipolysis in human adipocytes, suggesting that besides regulating LD morphology there is an additional level of regulation of lipolysis by FSP27.

An important aspect of the present study is that our results are relevant to human physiology, as all experiments were performed using cultured human adipocytes. Our studies in laboratory show that FSP27 interacts with ATGL (Grahn et al., in preparation). We found that FSP27 depletion causes an increase in both basal and stimulated lipolysis in human adipocytes (Fig. 2). In order to study the gain of function, we overexpressed ATGL and/or FSP27 in COS7 cells and mature human adipocytes. Interestingly, FSP27 expression suppressed ATGL-mediated lipoysis in both the model systems, indicating a direct role of FSP27 in regulating ATGL-mediated lipolsis.

This study did not address whether FSP27 interacts with ATGL directly or indirectly and whether it interacts with ATGL on the lipid droplet surface. Since FSP27 and ATGL are present in other compartments like the endoplasmic reticulum in adipocytes, it is possible that FSP27 interacts with ATGL in these compartments as well. Nonetheless, our study clearly shows the role of FSP27 in the regulation of ATGL-mediated lipolysis in human adipocytes.

The molecular mechanism whereby FSP27 regulates ATGL-mediated lipolysis is unknown. Adipocyte lipolysis is initiated when catecholamines bind to β-adrenergic receptors on the surface of a cell (23). Through the action of a stimulatory G-protein, adenylate cyclase is activated, leading to increased

intracellular cAMP and activation of cAMP-dependent PKA. PKA phosphorylates both PLIN1 and HSL (23,24,25,26). It is the PKA-dependent phosphorylation of PLIN1 that causes the release of CGI-58, leading to the initiation of lipolysis by activating ATGL (7,25,27). In the present model of lipolysis, PLIN1 binds CGI-58 in the basal state, limiting its access to ATGL, but it is not clear what restrains ATGL from binding CGI-58. Complementary to the current model, we propose a potential model in which FSP27 interacts with ATGL at the surface of lipid droplet and limits its access to CGI-58.

Our recent study showed that FSP27 interacts with PLIN1 at the lipid droplet surface (21). We propose here that PLIN1 being a scaffolding protein, interacts with FSP27 and helps it to stay and interact with ATGL at the droplet surface, but that only ATGL directly interacts with FSP27 (but not with PLIN1). In contrast, PLIN1 directly interacts with CGI-58 (28,29). However, what is not known is if the domain of ATGL that FSP27 binds to is the “active” site that is bound by CGI-58 to activate lipolysis. Our model is consistent with our data that FSP27 depletion increases lipolysis in human adipocytes under both basal and stimulated conditions, which is probably due to the release of FSP27-interacting with ATGL, which now gains an access to CGI-58. We cannot ignore the possibility that FSP27 regulation of LD morphology can add to the process of ATGL-regulated liplysis. But this hypothesis remains to be tested.

Our study also tested the possibility of the regulation of ATGL expression by FSP27. Surprisingly, knock down of FSP27 increased the expression of ATGL in human adipocytes. In contrast, the overexpression of FSP27 decreased ATGL expression. These studies clearly show that besides regulating the activity of ATGL, FSP27 could also regulate the transcription and translation of ATGL. Future studies are needed to describe the molecular mechanism of FSP27 regulation of ATGL expression. Overall, our studies show that FSP27 regulates the lipolysis in human adipocytes by regulating the activity as well as expression of ATGL.

FSP27 has previously been shown to play a role in TG accumulation in adipocytes (12,15,30). Precisely how FSP27 regulates TG accumulation is unclear. Based upon our results showing FSP27-ATGL interaction and regulation of ATGL-mediated liplysis by FSP27, we hypothesize that FSP27-mediated TG accumulation is due to its role in lipolysis regulation, i.e. FSP27 reduces lipolysis of stored TGs to increase their content. Indeed, our results showed that FSP27-mediated TG accumulation in human adipocytes was impaired upon ATGL depletion. Furthermore, when ATGL was depleted from cells, there was a significant decrease in basal lipolysis, however, when FSP27 was co-expressed in these ATGL-depleted cells, there was no further decrease in lipolysis, which suggests that FSP27-mediated regulation of lipolysis and TG accumulation was specifically ATGL-dependent.

LIST OF JOURNAL ABBREVIATIONS

Am. J. Physiol. Endocrinol. Metab.	American Journal of Physiology - Endocrinology and Metabolism
Am. J. Physio. Reg. Integ. & Com. Ph.	American Journal of Physiology Regulatory, Integrative and Comparative Physiology
Cell Metab.	Cell Metabolism
EMBO Mol. Med.	European Molecular Biology Organization – Molecular Medicine
J. Biol. Chem.	Journal of Biological Chemistry
J. Cell Biol.	The Journal of Cell Biology
J. Clin. Invest.	Journal of Clinical Investigation
J. Lipid Res.	Journal of Lipid Research
Mol. Endo.	Molecular Endocrinology
PLoS One	Public Library of Science
Prog. Lipid Res.	Progress in Lipid Research
Proc. Natl. Acad. Sci.	Proceedings of the National Academy of Sciences
Proc. Nutr. Soc.	Proceedings of the Nutrition Society

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