Small Ruminant Research 153 (2017) 1–4

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Short communication

Comparative sequence analysis and adipose-specific expression of G0S2 and ATGL in sheep

Jinsoo Ahn[a], Huiguang Wu[a,b], Yeunsu Suh[a], Ana C. Carranza[c], Alejandro E. Relling[c], Sangsu Shin[d], Sang Suk Lee[a,e], Kichoon Lee[a,*]

[a] Department of Animal Sciences, The Ohio State University, Columbus, OH 43210, USA
[b] School of Life Science, Inner Mongolia University for Nationalities, Huolinhe Street West No. 536, Tongliao 028000, China
[c] Department of Animal Sciences, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691, USA
[d] Department of Animal Biotechnology, Kyungpook National University, Sangju 37224, Republic of Korea
[e] Department of Animal Science and Technology, Sunchon National University, Suncheon 57922, Republic of Korea

ARTICLE INFO

Keywords:
Lipolysis
Adipose triglyceride lipase (ATGL)
G0/G1 switch gene 2 (G0S2)
Sheep
Hydrophobic domain
Adipose-specific expression

ABSTRACT

Lipolysis is a catabolic reaction that is mediated by adipose triglyceride lipase (ATGL) and its inhibitor such as G0/G1 switch gene 2 (G0S2) protein during the first step of hydrolysis. The hydrophobic domain of G0S2 protein interacts and binds to the patatin-like domain in ATGL. Although conservation of the hydrophobic domain has been reported across species, sheep G0S2 amino acids and its hydrophobic domain have not been sequenced and studied. In this study, the hydrophobic region of G0S2 which plays an important role in inhibition of ATGL-mediated lipolysis was highly conserved as shown in other species. Phylogenetic analysis revealed that ruminant G0S2 proteins showed greater homologies within the ruminant species compared to other mammalian species. Moreover, adipose-specific expression of *G0S2* for sheep was observed. Together with an abundant expression of ATGL protein in adipose tissue of sheep, G0S2 protein may play a critical role in inhibiting ATGL-mediated triacylglycerol (TAG) hydrolysis in the adipose tissue.

1. Introduction

Lipolysis is a sequential catabolic reaction mediated by lipolytic enzymes including adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MGL). G0/G1 switch gene 2 (G0S2) protein has been known to be involved in the first rate-limiting step through inhibition of ATGL (Yang et al., 2010). The hydrophobic domain of G0S2 protein interacts and binds to the patatin-like domain in ATGL (Yang et al., 2010). Conservation of the hydrophobic domain of G0S2 across species has been reported in several studies (Ahn et al., 2013; Ahn et al., 2014). However, sheep G0S2 has not been sequenced and evolutionary conservation of its hydrophobic domain has not been studied.

In this study, G0S2 conservation in sheep was examined based on our deposited sequence, and the relationship between G0S2 of ruminant species was revealed using phylogenetic analysis. In addition, adipose tissue-specific expression of sheep G0S2 along with sheep ATGL expression was investigated. These studies provide insight into the role of G0S2 in ATGL-mediated lipolysis in sheep.

2. Materials and methods

2.1. Animal preparation

All animal procedures used in these studies were approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC # 2016A00000013). Healthy adult sheep have been used to sequence the *G0S2* gene. For investigating the tissue distribution of *G0S2* and *ATGL*, the adipose tissue, heart, muscle, lung, liver, kidney, and spleen were harvested from healthy adult sheep (7–8-month-old) after slaughter (N = 4).

2.2. Sequencing

Adipose tissue from adult sheep was collected and snap-frozen in liquid N_2 before storing at −80° C. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Reverse transcription was conducted to synthesize cDNA using 1 μg of total RNA, oligo dT and the Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a 20 μl reaction volume and

* Corresponding author at: 2029 Fyffe Road, Columbus, OH 43210, USA.
E-mail address: lee.2626@osu.edu (K. Lee).

http://dx.doi.org/10.1016/j.smallrumres.2017.05.001
Received 2 November 2016; Received in revised form 3 February 2017; Accepted 3 May 2017
Available online 06 May 2017
0921-4488/

with conditions of 65 °C for 5 min, 37 °C for 52 min, and 70 °C for 15 min. PCR was performed using the newly synthesized cDNAs as a template to amplify the coding sequence of sheep *G0S2*. Several combinations of primers designed previously based on bovine *G0S2* sequence (GenBank accession number NM_001192147.1) were tested, and a set of a forward primer (5′-CAG CCC AGA TGG AGA CGG T-3′) and a reverse primer (5′-TGG TAG TTC AGT TCT AGA AGT CGG TGG-3′) amplified sheep *G0S2*. The PCR reactions were carried out in a MJ Research PTC-200 thermal cycler (MJ Research Inc., South San Francisco, CA) using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) and conditions of 10 min at 95 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, extension at 72 °C for 1 min, and a final extension of 10 min at 72 °C. Gel electrophoresis on a 1% agarose gel was used to separate the PCR products. The only one amplified band of the sheep *G0S2* was excised. DNA was extracted using a Qiagen Gel Extraction Kit (Qiagen, Alameda City, CA) and was sequenced by The Ohio State University Sequencing Core Facility using an Applied Biosystems 3730 DNA analyzer (Foster City, CA).

2.3. Phylogenetic tree

The G0S2 amino acid sequences other than the sheep sequence were derived from a Uniprot database (http://www.uniprot.org/) and Protein database (https://www.ncbi.nlm.nih.gov/protein). Sequence alignments were performed by using the ClustalX multiple alignment software (Thompson et al., 1994) in BioEdit Sequence Alignment Editor (Version 7.0.9.0; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap tests using the MEGA 6 software (Tamura et al., 2013).

2.4. Real-time PCR measurement of gene expression

Real-time PCR was conducted to assess relative gene expression using AmpliTaq Gold polymerase (Applied Biosystems) and SYBR green I as a detection dye for a dissociation curve on the ABI 7300 Real-Time PCR instrument (Applied Biosystems). To perform real-time PCR for sheep *G0S2*, forward (5′-GAA GGC GAG GAG AAG CAG AA-3′) and reverse (5′-CAG GTA CAT TCG CAC CAG CTT-3′) primers were designed on the first and second exons of the sheep *G0S2* sequence (XM_015099392.1), respectively, to avoid possible genomic DNA amplification. Primers for sheep *ATGL* were 5′-GTG GAC GGT GGC ATC TCA GA −3′(forward) and 5′-GCA GGT GCT CCA GGA TGT GA-3′(reverse), sheep *CYC* were 5′-GTG GTC ATC GGT CTC TTT GG-3′ (forward) and 5′-CAC CGT AGA TGC TCT TAC CTC-3′(reverse), sheep *GAPDH* were 5′- TCC CTG AGC TCA ACG GGA A-3′ (forward) and 5′-GCC GAA TTC ATT GTC GTA CCA-3′ (reverse), and sheep *β-actin* were 5′-CCA ACC GTG AGA AGA TGA CC-3′ (forward) and 5′-CCA GAG GCG TAC AGG GAC AG-3′ (reverse). Thermal profile was as follows: 95 °C for 10 min, 45 cycles of 94 °C for 15 s, 56 °C for 40 s, 72 °C for 30 s, and 82 °C for 32 s. Gene stability values of sheep *CYC*, *GAPDH*, and *β-actin* were estimated using the NormFinder software (Andersen et al., 2004) after performing real-time PCR. The geometric mean of the expression levels of *CYC* and *GAPDH* was used as a reference because combination of these genes showed the best stability value, as previously reported (Vandesompele et al., 2002). The relative gene expression level of target genes was calculated using the C_T values for the internal control and target genes, as measured by the ABI software, and the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

2.5. Western blot analysis

Western blot analysis with tissue protein extracts was performed as described in our previous report (Ahn et al., 2015). In particular, equal amounts of protein extracts were loaded onto gels before wet-transfer to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked for 30 min and then incubated with ATGL antibody (2138S; 1:1000; Cell Signaling Technology, Beverly, MA) at 4 °C overnight. The next day, after washing, an appropriate secondary antibody (HRP-linked anti-rabbit IgG (HAF008); 1:5000; R & D systems Inc., Minneapolis, MN) was applied to the membrane before washing and developing with ECL plus reagents and X-ray films (both materials from GE Healthcare Biosciences, Pittsburgh, PA). Commercial G0S2 antibodies that were tested in this study (H00050486-B01P; Abnova Inc., Walnut, CA, and sc-133423 and sc-133424; SantaCruz Biotechnology, Santa Cruz, CA) were not capable of detecting sheep G0S2 protein. The amount of protein extracts for Coomassie staining was used as a loading control.

2.6. Statistical analysis

Statistical analyses were conducted by using statistical software programs as follows: One-way ANOVA built in the SAS software program (SAS Institute, Inc.) was used to compare multiple means followed by Tukey's post-hoc test. *P*-value was < 0.05 for the minimum level of significance.

3. Results

The sequencing analysis revealed that the coding sequence of sheep *G0S2* is 261 bp. This nucleotide sequence has been deposited in GenBank (accession number JN935379.1). From the nucleotide sequence, putative sheep G0S2 amino acid sequence were predicted using the ORF finder in the Sequence Manipulation Suite website (http://www.bioinformatics.org/sms2/orf_find.html). The putative hydrophobic domain located between residues 27 and 42 was conserved substantially in sheep with a few amino acid variations compared to

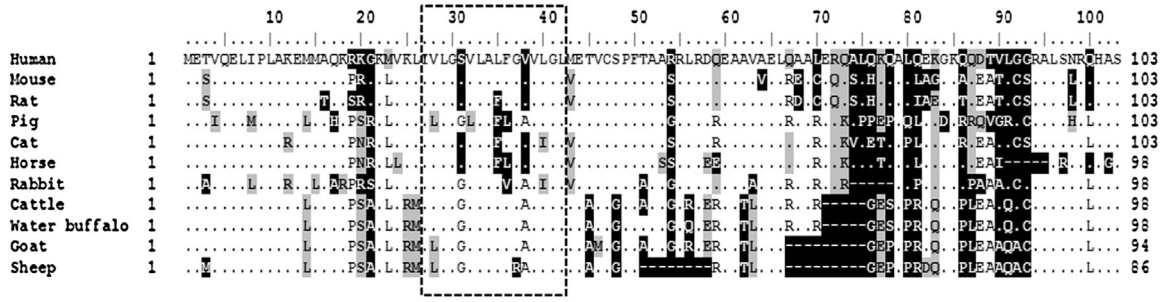

Fig. 1. Multiple sequence alignment of G0S2 amino acids from various species: human (GenBank accession number: NP_056529.1), mouse (NP_032085.1), rat (NP_001009632.1), pig (NP_001273733.1), cat (XP_011289272.1), horse (XP_005609886.1), rabbit (XP_002717587.1), cattle (NP_001179076.1), water buffalo (XP_006055219.1), goat (XP_017916268.1), and sheep (AFS34516.1). *Grey shading* represents amino acids containing the R group with similar properties, *black boxes* indicate substitutions of amino acids with the different R groups, and *black boxes with dashes* indicate deletions. *Dots* represent amino acids that are identical to human G0S2 protein. The hydrophobic domain located between residue 27 and 42 is marked with *a dashed square*.

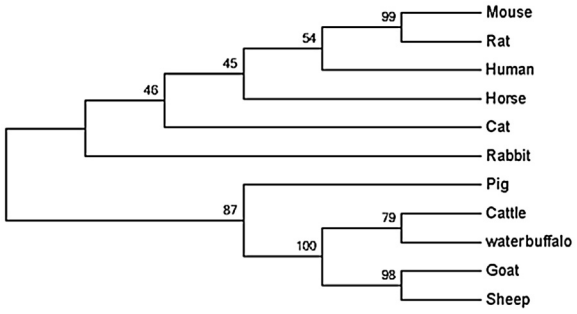

Fig. 2. A phylogenetic tree of G0S2 proteins constructed by the neighbor-joining method. Note a distinct hierarchical structure formed in a ruminant cluster composed of cattle, water buffalo, goats, and sheep indicating the relation between ruminant G0S2 sequences. The numbers above the branches indicate percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates).

humans and mice (Fig. 1). Among variations, Ser to Gly at residue 31 and Val to Ala at residue 38 were also shown in other ruminants such as cattle, water buffalo, and goats. Val to Leu substitution at residue 28 was present in both goats and sheep. Gly to Arg substitution at residue 37 was found only in sheep. The amino acid variations of Val to Leu (residue 28) and Val to Ala (residue 38) are substitutions between amino acids with hydrophobic side chains.

Compared to G0S2 amino acid sequences from humans and mice, ruminants have deletions in a non-conserved area around amino acid residue 67–93, resulting in relatively shorter G0S2 proteins (Fig. 1). Among ruminants, cattle, and water buffalo have deletions of 5 consecutive amino acids. Both goats and sheep have a deletion of 9 amino acids at the same position (67–76) and sheep have an additional deletion of 8 amino acids at the position (51–58), resulting in a total of 86 amino acids in sheep G0S2 protein. A phylogenetic analysis revealed that G0S2 proteins from ruminant species including cattle, water buffalo, goats, and sheep shared high homologies (Fig. 2). In addition, ruminant G0S2 proteins showed greater homologies to pigs compared to the other species.

Multiple alignment of ATGL amino acid sequences of sheep and other mammals (humans, mice, and pigs) and ruminants (cattle and goats) showed that the two main domains in ATGL, the patatin-like domain (residues 10–178) and the hydrophobic domain (residues 309–396), are conserved among the species. In the patatin-like domain, the glycine-rich, serine lipase motif (*GXGXXG*) and the active serine hydrolase motif (*GXSXG*) were identical as GCGFLG and GASAG, respectively, among the species (Supplementary Fig. 1).

Real-time PCR revealed that the expression of sheep *G0S2* is significantly higher in adipose tissue compared to other tissues (Fig. 3A). Adipose tissue showed the highest relative gene expression of *ATGL* (Fig. 3B). Protein extracted from adipose tissues of mice, cattle, and sheep were subjected to Western blot analysis and the expected ATGL bands were detected around 50 kDa (Fig. 3C). Western blot analysis revealed that the ATGL protein level in adipose tissue was much higher than other tissues (Fig. 3D).

4. Discussion

G0S2 protein binds through its hydrophobic domain to the patatin-like domain in ATGL possessing triacylglycerol (TAG) hydrolase activity (Yang et al., 2010) and this hydrophobic domain was largely conserved in sheep. Recent studies with deletion mutants of human G0S2 protein showed a complete inhibition of ATGL activity with a minimum area of G0S2 protein (residues 10–43) and a significant reduction of inhibition when the hydrophobic domain was fragmented (Cerk et al., 2014). This suggests that the first half of G0S2 protein is essential and sufficient in inhibiting ATGL activity. Therefore, conservation of the N-terminal area containing the hydrophobic domain supports biological and evolutionary advantage in maintaining the protein function across species including sheep. Whereas, variations and deletions in the second half of sheep G0S2 protein could be evolutionarily allowed without disrupting functional activity of sheep G0S2 protein.

Evolutionary relationship of G0S2 proteins between ruminant species were determined by phylogenetic analysis as indicated in a distinct hierarchical structure formed in a ruminant cluster. Also, ruminant G0S2 showed smaller evolutionary distance to G0S2 of porcine species than that of other mammalian species.

Conservation of the two important domains in sheep ATGL suggests the evolutionary importance in maintaining the TAG hydrolase (lipase) activities via the patatin-like domain as well as association with the surface of lipid droplets (LDs) through the hydrophobic domain. The enzymatic activity of ATGL was maintained with residues 1–254 fragment of ATGL and this could be due to the proper patatin fold (Cornaciu et al., 2011). In this regard, the high homology of sheep ATGL in the region of residues 1–254 compared to ATGL from other species suggests it retains the hydrolase activity. The hydrophobic domain (residues 309–396) is rich in hydrophobic amino acids and is involved in localization of ATGL to LDs (Fischer et al., 2007). Thus, the high conservation of the hydrophobic domain of sheep ATGL suggests its lipid droplet association.

Bovine and porcine *ATGL* levels in various tissues have been reported by our group (Deiuliis et al., 2008; Deiuliis et al., 2010). The high *ATGL* mRNA expression in sheep adipose tissue in this study was consistent with those expressions in bovine and porcine species. Moreover, ATGL protein expression exclusively in adipose tissue was consistent with cattle and pig ATGL protein expression as shown in our reports (Deiuliis et al., 2008; Deiuliis et al., 2010). Given that ATGL provides its TAG hydrolase activities through localization to LDs (Smirnova et al., 2006; Yang et al., 2010), abundant sheep ATGL expression in fat cells containing LDs was anticipated and it was shown in this study. In addition, the highest mRNA expression of *G0S2* in adipose tissue of porcine and bovine species (Ahn et al., 2013; Ahn et al., 2014) remained similar tissue distribution in sheep. Taken together, even though G0S2 protein expression was not detected using antibodies in our stock, a high level of G0S2 protein expression in adipose tissue may support the role of G0S2 protein in inhibition of ATGL.

In conclusion, the sheep *G0S2* sequenced in this study was highly conserved in the hydrophobic region, suggesting the role of G0S2 protein in inhibition of ATGL through protein–protein interactions. Other ruminant G0S2 proteins were largely homologous to sheep G0S2 protein according to phylogenetic analysis. High expression of *ATGL* and *G0S2* in adipose tissue from sheep suggests a critical interplay between ATGL and G0S2 proteins in fat cells. Future research will need to elucidate the regulation of sheep ATGL and G0S2 in adipose tissue under various developmental conditions and during nutritional interventions.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

This research was partially supported by the Ohio Agricultural Research and Development Center Interdisciplinary Research SEEDS funding, the Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ010906), RDA, Republic of Korea, Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2016R1C1B1013407), the China Scholarship Council (Grant No. 201508155023), and the National Nature Science Foundation of China (Grant No. 31402106).

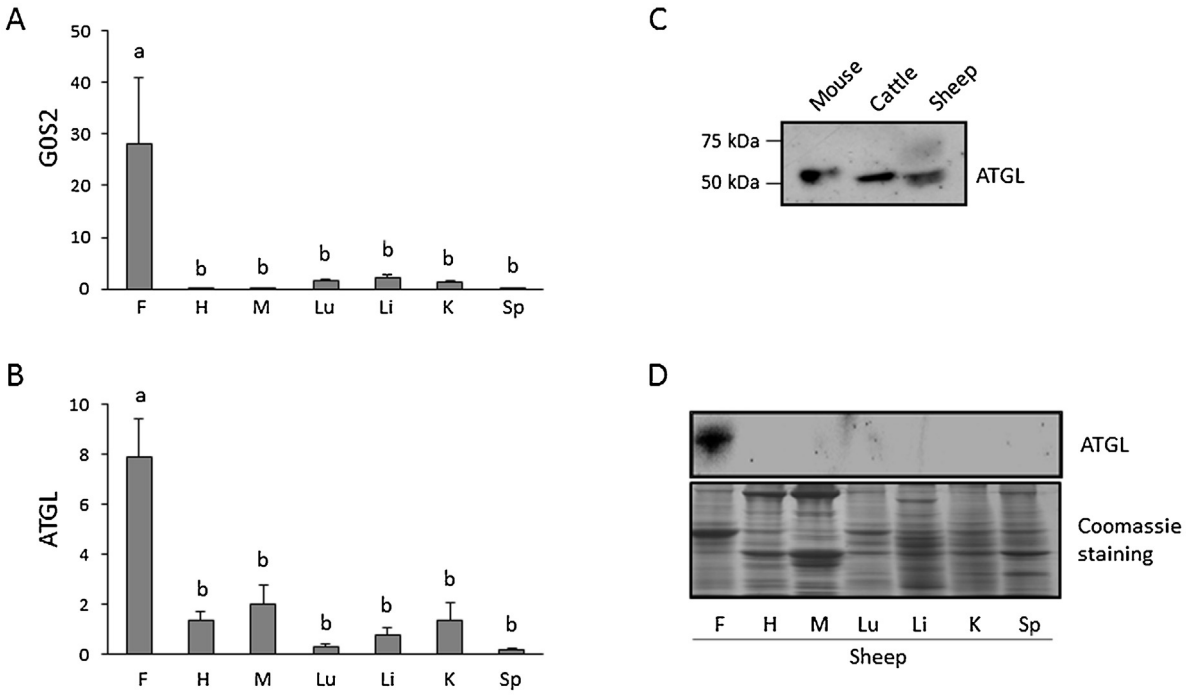

Fig. 3. (A and B) Measurements of sheep *G0S2* and *ATGL* mRNA expression levels using real-time PCR (N = 4). The geometric mean of the expression levels of *CYC* and *GAPDH* was used as a reference. (C) Protein lysates from adipose tissues of mice, cattle, and sheep were separated by SDS-PAGE and immunoprobed with the ATGL antibodies. (D) Western blot analysis of sheep ATGL in various tissues. Coomassie staining was used as a loading control. Adipose tissue or fat (F), heart (H), muscle (M), lung (Lu), liver (Li), kidney (K), and spleen (Sp).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.smallrumres.2017.05.001.

References

Ahn, J., Oh, S.A., Suh, Y., Moeller, S.J., Lee, K., 2013. Porcine G_0/G_1 switch gene 2 (G0S2) expression is regulated during adipogenesis and short-term in-vivo nutritional interventions. Lipids 48 (3), 209–218.

Ahn, J., Li, X., Choi, Y.M., Shin, S., Oh, S.A., Suh, Y., Nguyen, T.H., Baik, M., Hwang, S., Lee, K., 2014. Differential expressions of G0/G1 switch gene 2 and comparative gene identification-58 are associated with fat content in bovine muscle. Lipids 49 (1), 1–14.

Ahn, J., Shin, S., Suh, Y., Park, J.Y., Hwang, S., Lee, K., 2015. Identification of the avian RBP7 gene as a new adipose-specific gene and RBP7 promoter-driven GFP expression in adipose tissue of transgenic quail. PLoS One 10 (4), e0124768.

Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64 (15), 5245–5250.

Cerk, I.K., Salzburger, B., Boeszoermenyi, A., Heier, C., Pillip, C., Romauch, M., Schweiger, M., Cornaciu, I., Lass, A., Zimmermann, R., Zechner, R., Oberer, M., 2014. A peptide derived from G0/G1 switch gene 2 acts as noncompetitive inhibitor of adipose triglyceride lipase. J. Biol. Chem. 289 (47), 32559–32570.

Cornaciu, I., Boeszoermenyi, A., Lindermuth, H., Nagy, H.M., Cerk, I.K., Ebner, C., Salzburger, B., Gruber, A., Schweiger, M., Zechner, R., Lass, A., Zimmermann, R., Oberer, M., 2011. The minimal domain of adipose triglyceride lipase (ATGL) ranges until leucine 254 and can be activated and inhibited by CGI-58 and G0S2, respectively. PLoS One 6 (10), e26349.

Deiuliis, J.A., Shin, J., Bae, D., Azain, M.J., Barb, R., Lee, K., 2008. Developmental, hormonal, and nutritional regulation of porcine adipose triglyceride lipase (ATGL). Lipids 43 (3), 215–225.

Deiuliis, J., Shin, J., Murphy, E., Kronberg, S.L., Eastridge, M.L., Suh, Y., Yoon, J.T., Lee, K., 2010. Bovine adipose triglyceride lipase is not altered and adipocyte fatty acid-binding protein is increased by dietary flaxseed. Lipids 45 (11), 963–973.

Fischer, J., Lefèvre, C., Morava, E., Mussini, J.M., Laforêt, P., Negre-Salvayre, A., Lathrop, M., Salvayre, R., 2007. The gene encoding adipose triglyceride lipase (PNPLA2) is mutated in neutral lipid storage disease with myopathy. Nat. Genet. 39 (1), 28–30.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25, 402–408.

Smirnova, E., Goldberg, E.B., Makarova, K.S., Lin, L., Brown, W.J., Jackson, C.L., 2006. ATGL has a key role in lipid droplet/adiposome degradation in mammalian cells. EMBO Rep. 7 (1), 106–113.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30 (12), 2725–2729.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22 (22), 4673–4680.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 (7) RESEARCH0034.

Yang, X., Lu, X., Lombes, M., Rha, G.B., Chi, Y.I., Guerin, T.M., Smart, E.J., Liu, J., 2010. The G0/G1 switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. Cell Metab. 11, 194–205.