# Cloning and functional characterization of porcine AACS for subcutaneous fat deposition

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# Cloning and functional characterization of porcine AACS for subcutaneous fat deposition

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## Abstract

Fat deposition is a quantitative trait controlled by multiple genes in pigs. Using transcriptome sequencing, we previously reported that *AACS* is differentially expressed in the subcutaneous fat tissue of Dingyuan pigs with divergent back fat thickness. Therefore, with the aim of further characterizing this gene and its protein, we cloned the entire 3286-bp mRNA sequence of the porcine *AACS,* and the encoded AACS protein is a hydrophilic protein without a signal peptide or transmembrane sequence. Our findings suggested that among various tissues and pig breeds, *AACS* was highly expressed in subcutaneous fat. We identified three fully-linked SNP sites, A-1759C, C-1683T, and A-1664G, in *AACS* that may be vital molecular markers for regulating back fat thickness. The double luciferase activity test in the 5′ flanking region indicated that the flanking region of *AACS* contained several active regulatory elements. Finally, we observed that *AACS* overexpression inhibited the proliferation and differentiation of subcutaneous preadipocytes. Collectively, our results suggest that *AACS* inhibits subcutaneous fat deposition in pigs. This study provides a new molecular marker for understanding the mechanism of porcine fat deposition.

## Introduction

The main adipose tissue depot in pigs is subcutaneous adipose tissue (SAT), accounting for over 70 % of the total body fat in pigs[1]. In the actual production process, the excessive accumulation of subcutaneous fat increases production costs and reduces product quality, thus affecting economic benefits. Subcutaneous fat reduction is a critical aspect of pig breeding[2,3]; therefore, candidate genes that control subcutaneous fat deposition must be investigated.

Acetoacetyl-CoA synthase, also known as acetylacetic acid CoA (*AACS*), is a cytoplasmic ketone body (acetylacetic acid)-specific ligase found in various adipogenic tissues[4] that specifically converts acetylacetic acid to acetoacetyl-CoA for the synthesis of cholesterol and fatty acids[5]. *AACS* mRNA is especially abundant in white adipose tissue, and its expression increases during adipocyte differentiation[6]. The expression pattern of *AACS* during preadipocyte differentiation

is highly similar to that of acetyl-CoA carboxylase 1 (ACC-1), a key enzyme in fatty acid synthesis, suggesting that the function of *AACS* is related to fatty acid synthesis[6-8]. The C/ EBP-α binding site was found in the *AACS* promoter region, suggesting that (C/ EBP-α) is crucial for *AACS* expression in adipocyte differentiation[9]. *AACS* plays a vital role in the regulation of fat deposition.

We previously identified *AACS* as the key differentially expressed gene in the subcutaneous fat tissue of Dingyuan (DY) pigs with divergent back fat thicknesses using transcriptomic profiles[10]. Therefore, we surmised that *AACS* is a crucial candidate gene for back subcutaneous fat thickness in pigs. Accordingly, we cloned porcine *AACS* to evaluate its role in regulating fat deposition and identify its functional molecular markers. We believe that our results would help identify major genes or markers that may benefit the pig-breeding industry.

## Materials & Methods

## Ethics statements

Animal rearing and handling were performed in accordance with the Guide for the Care and Use of Laboratory Animals of China. All experimental protocols were approved by the Committee on the Ethics of Animal Experiments of the China Agricultural University (permit number: SKLAB-2012-04-07).

**Experimental materials**

Ear tissue samples were collected from Tibetan (TP, n = 34), Landrace (LL, n = 34), Yorkshire (YY, n = 40), and Berkshire Dingyuan (BD, n = 104) pigs for DNA extraction. The TP, LL, and YY pigs were raised at the Tibet Agriculture and Animal Husbandry College, and the BD pigs were raised at the Ankang Agriculture and Animal Husbandry Company, Dingyuan County, Anhui Province. BD individuals had phenotypic data of back fat thickness and were day-old at a body weight of 70 kg. RNA was extracted from the subcutaneous fat tissue and longissimus dorsi muscle of six-month-old TP (n = 6), YY (n = 6), and DY (n = 6). Six-day-old piglets were used to isolate primary preadipocytes. Animals were allowed access to food and water ad libitum and were maintained under the same conditions. The diet containing 13.5 MJ/kg of metabolizable energy and 16.0% crude protein. The pig ear samples were obtained as a general breeding monitoring procedure, after which the pigs continue to be fed at their original breeding base. Animals used to collect back adipose tissue for RNA extraction and cell isolation were slaughtered in accordance with Operating procedures of livestock and poultry slaughtering—Pig (GB/T 17236-2019，China). The pigs were fasted for 12 h with free access to fresh water. Then, pigs were stunned electrically at 90 V and 50 Hz for 10 s, and exsanguinated as essential to ameliorate pain.

**DNA, RNA, and cDNA preparation**

Genomic DNA was isolated from the ear tissue using a standard phenol/chloroform extraction method. Total RNA was extracted from tissue samples with TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA). Its quality and integrity were verified using a NanoDrop 2000 Biophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoresis. RNA samples (2 μg) in a 20 μL reaction volume were reverse transcribed to cDNA using the FastQuant Reverse Transcriptase Kit (TIANGEN, Beijing, China).

**Cloning and sequence analysis of AACS**

The 5′- and 3′-end sequences of the cDNA encoding the *AACS* were obtained from the adipose tissue of TP using the Smarter™ RACE cDNA Amplification kit 5′/3′ according to the manufacturer's instructions, and the remaining sequences were amplified using PCR; the specific primer sequences are listed in Table S1. All PCR products were gel purified and cloned into the PUC19 vector (included in the SMARTER® RACE 5′/3′ kit). The recombinant plasmid was sequenced using SinoGenoMax, and the full-length porcine *AACS* mRNA sequence was ligated using DNAMAN8 software (Lynnon Biosoft). The amino acid hydrophobicity was predicted using the Prot Scale in ExPASy (https://web.expasy.org/protscale/). ExPASy online software (https://web.expasy.org/protparam/) was used to predict the theoretical pI, net charged residues, and instability index of *AACS*.

**Measurement of gene expression**

A semi-quantitative polymerase chain reaction was used to detect *AACS* expression in several pig tissues, and the assay was performed as previously described[11]. Total RNA was reverse transcribed to cDNA using the FastQuant RT Kit (TIANGEN, Beijing, China). Then, quantitative real-time PCR (qPCR) was performed using SYBR Green qPCR SuperMix (TIANGEN, Beijing, China). The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The specific primer sequences are presented in Table S2, and the housekeeping gene, β-actin, served as the positive control.

**SNP screening, genotyping, and correlation analysis**

Three pairs of primers for SNP screening of the *AACS* were designed using Primer Premier software (version 5.0; Premier Biosoft, Palo Alto, CA, USA) and are listed in Table S3. The amplicon sequences covered 2002-bp regions in the 5′-flanking (numbered starting from the start codon; the first base upstream of ATG was designated as −1, followed by sequential numbering until −2,164). A single bright band of the final product was considered as qualified and sent to SinoGenoMax for sequencing. Chromas Pro (Technelysium Pty) and DNAMAN (version 8.0) were used to analyze sequence variation.

**Dual-luciferase reporter assays**

To detect the *AACS* gene promoter region activity, the −2044–+116 fragment of this promoter region was amplified and then cloned into a pGL3-basic vector using homologous recombination. The primers used for amplification are listed in Table S4. At 48 h post-transfection, the cells were lysed in 100 μL of lysis buffer and then assayed for promoter activity using a dual-luciferase reporter assay system (Promega, Madison, WI, USA) on the PerkinElmer 2030 Multilabel Reader (PerkinElmer, Waltham, MA, USA). The Renilla luciferase signal was normalized to the firefly luciferase signal.

**Isolation and culture of subcutaneous preadipocytes**

Subcutaneous adipocytes were stripped from subcutaneous deposits in the neck and back of six-day-old piglets. The adipose tissue was cut into small pieces and digested with 1 mg/mL collagenase type I (Invitrogen, Carlsbad, CA, USA) at 37 °C for 60 min in a reciprocating shaker bath, followed by filtration. Preadipocytes were cultured in a growth medium (GM) containing

Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibco) and 1 % penicillin/streptomycin (PS, Gibco). When the primary cells reached 100 % confluence, adipogenesis was induced with a differentiation medium consisting of a growth medium, 0.5 mM isobutylmethylxanthine, 20 nM insulin, and 0.5 mM dexamethasone for two days. Subsequently, cells were cultured in a maintenance medium consisting of 5 μg/mL insulin in GM. All cells were maintained in a humidified atmosphere containing 5 % $CO_2$ at 37 °C.

**Plasmid construction, lentivirus packaging, and lentivirus infection**

To amplify the CDS region of the porcine *AACS*, the corresponding fragment was inserted into the EcoRI/BamHI site of the pLenti-CMV-EGFP-3FLAG-PGK-Puro vector (Obio Technology, Shanghai, China). Using Lipofectamine 2000-mediated transfection, 293T cells were co-transfected with the pLenti-CMV-EGFP-3FLAG-PGK-Puro vector, vesicular stomatitis virus G protein plasmid, or packaging plasmid. After 48 h, the cells generated mature lentivirus-containing supernatant. The preadipocytes were infected with the lentivirus and then screened with a concentration of 5 ug/mL of puromycin to obtain a stable *AACS*-overexpressed preadipocytes line.

**Oil Red O staining and dye extraction analysis**

After removing the growth medium, cells were fixed with 4 % paraformaldehyde for 30 min at room temperature. The cells were washed with PBS and stained with Oil Red O working solution (Sigma, St. Louis, MO, USA) for 30 min. The cells were again washed with PBS and observed under a microscope. Finally, Oil Red O dye was extracted from the stained cells with isopropanol for 20 min, and the lipid droplet content was evaluated by spectrophotometrically measuring the absorbance at 490 nm.

**CCK8 and EdU proliferation assays**

The cells were inoculated in 96-well plates, and 10 μL of CCK8 (Beyotime Biotechnology, Shanghai, China) solution was added to each well before the assay. After incubation for 1 h, the absorbance was measured at 450 nm using a microplate reader (Biotek, Winooski, VT, USA).

The EdU assay was performed using an EdU assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Cell nuclei and EdU-positive cells were stained blue and red, respectively.

**Statistical analysis**

Statistically significant differences were determined via the t-test using SPSS software (version 21.0; IBM, Chicago, IL, USA). Graphs were prepared using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA). The results are expressed as mean ± standard deviation (SD), with statistical significance set at $P < 0.05$ and highly significant values at $P < 0.01$.

## Results

**Cloning and sequence analysis of AACS**

The complete mRNA sequence of *AACS* was successfully cloned using 5′ rapid amplification of cDNA ends (RACE), 3′ RACE, and PCR (Figure 1a). The sequence with a 5′ end length of 1892

bp was obtained using nested PCR, with the first amplified product being 2211 bp. The sequence with a 3′ end length of 577 bp was obtained using 3′ RACE, and the intermediate sequence was obtained using PCR amplification with 1575 bp. After sequencing and assembling, the full-length 3286-bp *AACS* sequence (Accession Number, OP807955), including the coding sequence of 2019 bp, 5′ untranslated region of 161 bp, and 3′ untranslated region of 1106 bp, encoding a total of 672 amino acids; was obtained. Sequence analysis with ExPASy revealed that the isoelectric point of the AACS protein was 5.79, with a relative molecular mass of 75 kDa and an instability index of 32.82; therefore, it was presumed to be a stable protein. Hydrophobicity prediction analysis revealed strong hydrophilic regions in many parts of the protein. The hydrophobic regions were evenly distributed with an average value of −0.170, indicating that *AACS* is hydrophilic (Figure 1b). Furthermore, no signal peptide sequence or transmembrane domain was present in this gene (Figure 1c). The results indicated nucleotide similarities of 89.71, 89.14, 88.44, 88.15, and 86.68 % and amino acid sequence similarities of 93.9, 93.45, 91.82, 91.67, and 90.62 % for cattle, sheep, donkeys, horses, and humans, respectively, suggesting that the *AACS* is relatively conserved among species.

**Expression profile of porcine AACS in tissues**

The *AACS* expression in the lung, liver, kidney, leg, hypothalamus, back fat, heart, and longest dorsal (LD) muscle tissues of pigs were evaluated. The results revealed that *AACS* was expressed in all eight tissues, with high expression in the heart and back fat (Figure 2a). To investigate *AACS* function in fat deposition, *AACS* expression was examined in the BF and LD tissues. In BF tissue, *AACS* expression was significantly lower in TP than in YY tissues, and no significant differences were observed between these in LD tissue (Figure 2b). Similar results were obtained in DY pigs with divergent back fat thicknesses. In back fat tissue, *AACS* expression was significantly lower in the high back fat thickness (HBF) group than in the low back fat thickness (LBF) group, with no significant difference in LD tissue (Figure 2c). These results suggest that *AACS* might play a negative regulatory role in back fat deposition in pigs.

**SNP identification of 5′flanking region in AACS**

Using Sanger sequencing, three complete linkage mutation loci in the 5′ flanking region, namely A-1759C, C-1683T, and A-1664G, were identified (Figure 3). The three SNPs were completely linked and formed two haplotypes, ACA and CTG. Thus, the frequencies of allies and genotypes of A-1759C could represent the polymorphisms at these three sites. For site A-1759C, three genotypes (AA, AC, and CC) were observed in the LL and YY groups, and two genotypes (AA and AC) in the TP group, and the genotype frequency distribution was significantly different between the two lean-type (LL and YY) pigs and the TP group (Table 1). This linkage site is likely a crucial SNP marker associated with fat deposition.

To confirm this hypothesis, we genotyped A-1759C in 104 BD pigs and analyzed its association with back fat thickness and age up to 70 kg. The results helped identify 34, 58, and 12 pigs with the AA, AC, and CC genotypes, respectively. Association analysis showed that the back fat thickness of the AA and AC genotypes was significantly higher than that of the CC genotype ($P <$

0.01). No significant differences were observed between the three genotypes for ages up to 70 kg (Table 2).

**Transcription activity of AACS promoter region**

Four length fragments of the 5′ flanking region (−2044/+116, −1493/+116, −1050/+116, and −608/+116) were amplified, and the promoter activity in these four regions was detected using the dual-luciferase reporter assay. The results indicated that the fluorescence activity of these four sections was significantly higher than that of the control group (PGL3-control), indicating that these four sections contained critical regulatory elements. Among these, the −608/+116 region may have vital regulatory elements that enhance promoter activity, and the −1493/−2044 region may have critical active elements that inhibit promoter activity (Figure 4). The identified A-1759C, C-1683T, and A-1664G linkage sites were all located in the −1493/−2044 region; thus, their polymorphisms might affect the expression of *AACS* and fat deposition in pigs.

**AACS inhibits the proliferation of subcutaneous preadipocytes**

Porcine subcutaneous preadipocytes were infected with a lentivirus to overexpress *AACS*, and unsuccessfully infected cells were removed using puromycin. The results confirmed the successful overexpression of *AACS* using lentiviral transfection (Figure 5a). The CCK8 assay demonstrated that the number of living cells decreased significantly in the *AACS* overexpression group compared with that in the control group (Figure 5b). Similar results were obtained in the EDU assay. *AACS* overexpression resulted in reduced EdU positivity compared to the control group (Figure 5d). The mRNA levels of the representative proliferation markers, cyclin-dependent kinase 4 (*CDK4*) and cyclin B, decreased upon overexpression of *AACS* (Figure 5c), with cyclin B exhibiting significant differences.

**AACS inhibits adipogenic differentiation of subcutaneous preadipocytes**

To validate the *AACS*-mediated regulation of the differentiation of subcutaneous preadipocytes, Oil Red O staining was performed on day 6 of preadipocyte differentiation to detect the number of lipid droplets generated (Figure 6a). The results revealed that *AACS* overexpression in porcine preadipocytes significantly reduced the lipid droplet production (Figure 6b), and the expression of the marker genes of adipogenesis, *PPARγ*, *CEBPα*, *AP2*, and *SREBP-1C*, were significantly lower in the overexpression group than in the control group (Figure 6c). These results indicate that *AACS* plays an inhibitory role in the differentiation of subcutaneous preadipocytes in pigs.

## Discussion

This study is the first to clone *AACS* mRNA sequence and report that *AACS* plays an inhibitory role in the differentiation of porcine subcutaneous preadipocytes. These findings confirm the crucial role of *AACS* in porcine subcutaneous adipogenesis and proliferation. Our findings also elucidate the potential effect of *AACS* on the improvement of subcutaneous fat deposition and provide theoretical implications for its role in the improved breeding of pigs.

*AACS* directly activates ketone bodies in the cytosol to synthesize cholesterol and fatty acids[5]. We observed *AACS* expression in the adipose tissue of DY pigs with divergent back fat thicknesses, suggesting that *AACS* plays a role in the subcutaneous fat deposition in pigs. Porcine

*AACS* genes were cloned and compared using the NCBI for Biotechnology Information website. The high similarity of amino acid and nucleotide sequences between species indicates that the mRNA sequence of the cloned AACS gene is relatively accurate and that the gene is relatively conserved among species.

Highly conserved genes often share common regulatory mechanisms[12-14]. This study, along with others, demonstrated that *AACS* genes are highly expressed in adipose tissue[7] and play a negative regulatory role in the deposition of subcutaneous fat in pigs and that upregulation of *AACS* inhibits the differentiation of subcutaneous preadipocytes. In contrast, in 3T3-L1 cells, the downregulation of *AACS* inhibited cell differentiation[15]. Similar results have been found in mice, where the *AACS* gene expression level in white adipose tissue was lower in Zucker fatty rats than in lean rats. However, in high-fat, diet-induced obese rats, the expression levels of *AACS* were increased[7]. In the epididymal adipose tissue, *AACS* protein expression decreases in mice fed a short-term high-fat diet [16]. The expression trend of *AACS* in different species indicates that this is not only affected by obesity and species type but also by the location of fat deposition, where the mechanism of fat deposition differs at different locations[17-19]. The formation of adipose tissue mainly involves an increase in the number of adipocytes (proliferation) and their hypertrophy (differentiation)[20,21]. High expression of *AACS* inhibits the proliferation and differentiation of porcine subcutaneous preadipocytes, resulting in a decrease in flat droplet generation.

Active promoter elements with vital functions are present in the 5′ flanking region of the gene[22,23]. We detected the promoter activity of this region and observed that each flanker region gained strong promoter activity by truncating the amplified fragment, among which the −1493/−2044 region had important promoter-inhibiting active elements. Notably, an important linkage mutation site (A-1759C) was identified in this region, validated in a population of 104 pigs, and demonstrated to be significantly associated with back fat thickness, which may be a vital molecular marker for pig breeding.

In conclusion, we cloned porcine *AACS* mRNA sequences for the first time and confirmed that *AACS* is a negative regulator of subcutaneous preadipocyte differentiation in pigs. This study provides evidence that elevated *AACS* genes lead to the downregulation of PPARγ, CEBP/α, and AP2 genes which are key regulators of fat formation. Additionally, we explored luciferase activity in the 5′ flanking region of the *AACS* gene and obtained molecular markers that could be used for genetic improvement in pigs. Although we present preliminary findings, we confirmed that *AACS* is a vital regulator of porcine subcutaneous fat deposition and can have practical implications in improving pig breeding.

**Supplementary Materials:**

Table S1: The primer sequences for AACS gene cloning and SNP identification.

Table S2: The primer sequences for SqRT-PCR and qRT-PCR.

Table S3. The primer sequences for AACS gene SNP identification.

Table S4. The primer sequences for Dual-luciferase reporter assays.

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**Institutional Review Board Statement:** All experiments were approved by the Committee on the Ethics of Animal Experiments of China Agricultural University (permit number: SKLAB-2012-04-07).

**Informed Consent Statement:** Not applicable

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

Allen, C.E. Cellularity of adipose tissue in meat animals. *Federation proceedings* **1976**, *35*, 2302-2307.

Hocquette, J.F.; Gondret, F.; Baeza, E.; Medale, F.; Jurie, C.; Pethick, D.W. Intramuscular fat content in meat-producing animals: development, genetic and nutritional control, and identification of putative markers. *Animal : an international journal of animal bioscience* **2010**, *4*, 303-319, doi:10.1017/S1751731109991091.

Louveau, I.; Perruchot, M.H.; Bonnet, M.; Gondret, F. Invited review: Pre- and postnatal adipose tissue development in farm animals: from stem cells to adipocyte physiology. *Animal : an international journal of animal bioscience* **2016**, *10*, 1839-1847, doi:10.1017/S1751731116000872.

Ito, M.; Fukui, T.; Saito, T.; Tomita, K. Acetoacetyl-CoA synthetase specific activity and concentration in rat tissues. *Biochimica et biophysica acta* **1986**, *876*, 280-287, doi:10.1016/0005-2760(86)90285-7.

Endemann, G.; Goetz, P.G.; Edmond, J.; Brunengraber, H. Lipogenesis from ketone bodies in the isolated perfused rat liver. Evidence for the cytosolic activation of acetoacetate. *The Journal of biological chemistry* **1982**, *257*, 3434-3440.

6. Yamasaki, M.; Hasegawa, S.; Suzuki, H.; Hidai, K.; Saitoh, Y.; Fukui, T. Acetoacetyl-CoA synthetase gene is abundant in rat adipose, and related with fatty acid synthesis in mature adipocytes. *Biochem Bioph Res Co* **2005**, *335*, 215-219, doi:10.1016/j.bbrc.2005.07.053.

Yamasaki, M.; Hasegawa, S.; Kitani, T.; Hidai, K.; Fukui, T. Differential effects of obesity on acetoacetyl-CoA synthetase gene in rat adipose tissues. *Eur J Lipid Sci Tech* **2007**, *109*, 617-622, doi:10.1002/ejlt.200600265.

Yamasaki, M.; Hasegawa, S.; Yamanaka, H.; Narishima, R.; Fukui, T. Ketone Body Utilization is Regulated by Male-specific Factors in Rat Subcutaneous Adipocytes. *Exp Clin Endocr Diab* **2009**, *117*, 170-174, doi:10.1055/s-0028-1082073.

Hasegawa, S.; Yamasaki, M.; Inage, T.; Takahashi, N.; Fukui, T. Transcriptional regulation of ketone body-utilizing enzyme, acetoacetyl-CoA synthetase, by C/EBPalpha during adipocyte differentiation. *Biochimica et biophysica acta* **2008**, *1779*, 414-419, doi:10.1016/j.bbagrm.2008.05.001.

Zhang, P.; Li, Q.G.; Wu, Y.J.; Zhang, Y.W.; Zhang, B.; Zhang, H. Identification of candidate genes that specifically regulate subcutaneous and intramuscular fat deposition using transcriptomic

and proteomic profiles in Dingyuan pigs. *Sci Rep-Uk* **2022**, *12*, doi:Artn 2844, 10.1038/S41598-022-06868-3.

Wang, Z.; Li, Q.; Zhang, B.; Lu, Y.; Yang, Y.; Ban, D.; Zhang, H. Single nucleotide polymorphism scanning and expression of the FRZB gene in pig populations. *Gene* **2014**, *543*, 198-203, doi:10.1016/j.gene.2014.04.023.

Stuart, J.M.; Segal, E.; Koller, D.; Kim, S.K. A gene-coexpression network for global discovery of conserved genetic modules. *Science* **2003**, *302*, 249-255, doi:10.1126/science.1087447.

Gerstein, M.B.; Rozowsky, J.; Yan, K.K.; Wang, D.; Cheng, C.; Brown, J.B.; Davis, C.A.; Hillier, L.; Sisu, C.; Li, J.J.; et al. Comparative analysis of the transcriptome across distant species. *Nature* **2014**, *512*, 445-448, doi:10.1038/nature13424.

Chikina, M.D.; Troyanskaya, O.G. Accurate Quantification of Functional Analogy among Close Homologs. *Plos Comput Biol* **2011**, *7*, doi:ARTN e1001074.10.1371/journal.pcbi.1001074.

Hasegawa, S.; Ikeda, Y.; Yamasaki, M.; Fukui, T. The role of acetoacetyl-CoA synthetase, a ketone body-utilizing enzyme, in 3T3-L1 adipocyte differentiation. *Biological & pharmaceutical bulletin* **2012**, *35*, 1980-1985, doi:10.1248/bpb.b12-00435.

Plubell, D.L.; Wilmarth, P.A.; Zhao, Y.Q.; Fenton, A.M.; Minnier, J.; Reddy, A.P.; Klimek, J.; Yang, X.; David, L.L.; Pamir, N. Extended Multiplexing of Tandem Mass Tags (TMT) Labeling Reveals Age and High Fat Diet Specific Proteome Changes in Mouse Epididymal Adipose Tissue. *Mol Cell Proteomics* **2017**, *16*, 873-890, doi:10.1074/mcp.M116.065524.

Luo, N.; Shu, J.T.; Yuan, X.Y.; Jin, Y.X.; Cui, H.X.; Zhao, G.P.; Wen, J. Differential regulation of intramuscular fat and abdominal fat deposition in chickens. *Bmc Genomics* **2022**, *23*, doi:ARTN 30810.1186/s12864-022-08538-0.

Zhou, G.X.; Wang, S.B.; Wang, Z.G.; Zhu, X.T.; Shu, G.; Liao, W.Y.; Yu, K.F.; Gao, P.; Xi, Q.Y.; Wang, X.Q.; et al. Global comparison of gene expression profiles between intramuscular and subcutaneous adipocytes of neonatal landrace pig using microarray. *Meat Sci* **2010**, *86*, 440-450, doi:10.1016/j.meatsci.2010.05.031.

Mendizabal, J.A.; Soret, B.; Purroy, A.; Arana, A.; Horcada, A. Influence of sex on cellularity and lipogenic enzymes of Spanish lamb breeds (Lacha and Rasa Aragonesa). *Anim Sci* **1997**, *64*, 283-289, doi:Doi 10.1017/S135772980001585x.

Ghaben, A.L.; Scherer, P.E. Adipogenesis and metabolic health. *Nature reviews. Molecular cell biology* **2019**, *20*, 242-258, doi:10.1038/s41580-018-0093-z.

Choe, S.S.; Huh, J.Y.; Hwang, I.J.; Kim, J.I.; Kim, J.B. Adipose Tissue Remodeling: its Role in energy Metabolism and Metabolic Disorders. *Front Endocrinol* **2016**, *7*, doi:Artn 3010.3389/Fendo.2016.00030.

Li, P.Z.; Wang, B.; Cao, D.D.; Liu, Y.Z.; Zhang, Q.Q.; Wang, X.B. Characterization and functional analysis of the Paralichthys olivaceus prdm1 gene promoter. *Comp Biochem Phys B* **2017**, *212*, 32-40, doi:10.1016/j.cbpb.2017.06.009.

Kimura, N.; Tomizawa, S.; Arai, K.N.; Osamura, R.Y.; Kimura, N. Characterization of 5 '-flanking region of rat somatostatin receptor sst2 gene: Transcriptional regulatory elements and activation by Pitx1 and estrogen. *Endocrinology* **2001**, *142*, 1427-1441, doi:DOI 10.1210/en.142.4.1427.

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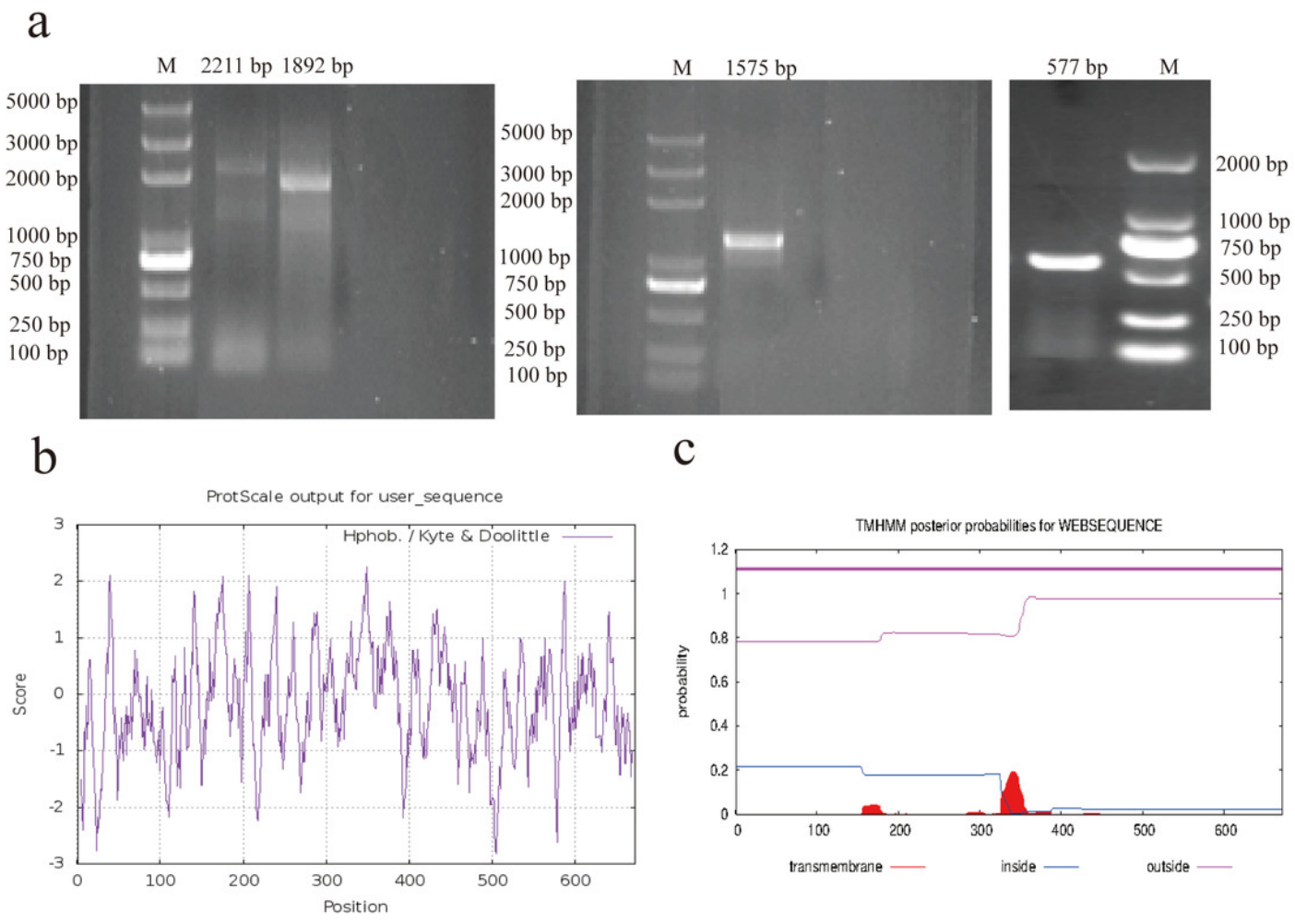
# Figure 1

Figure 1. RACE amplification and sequence analysis.

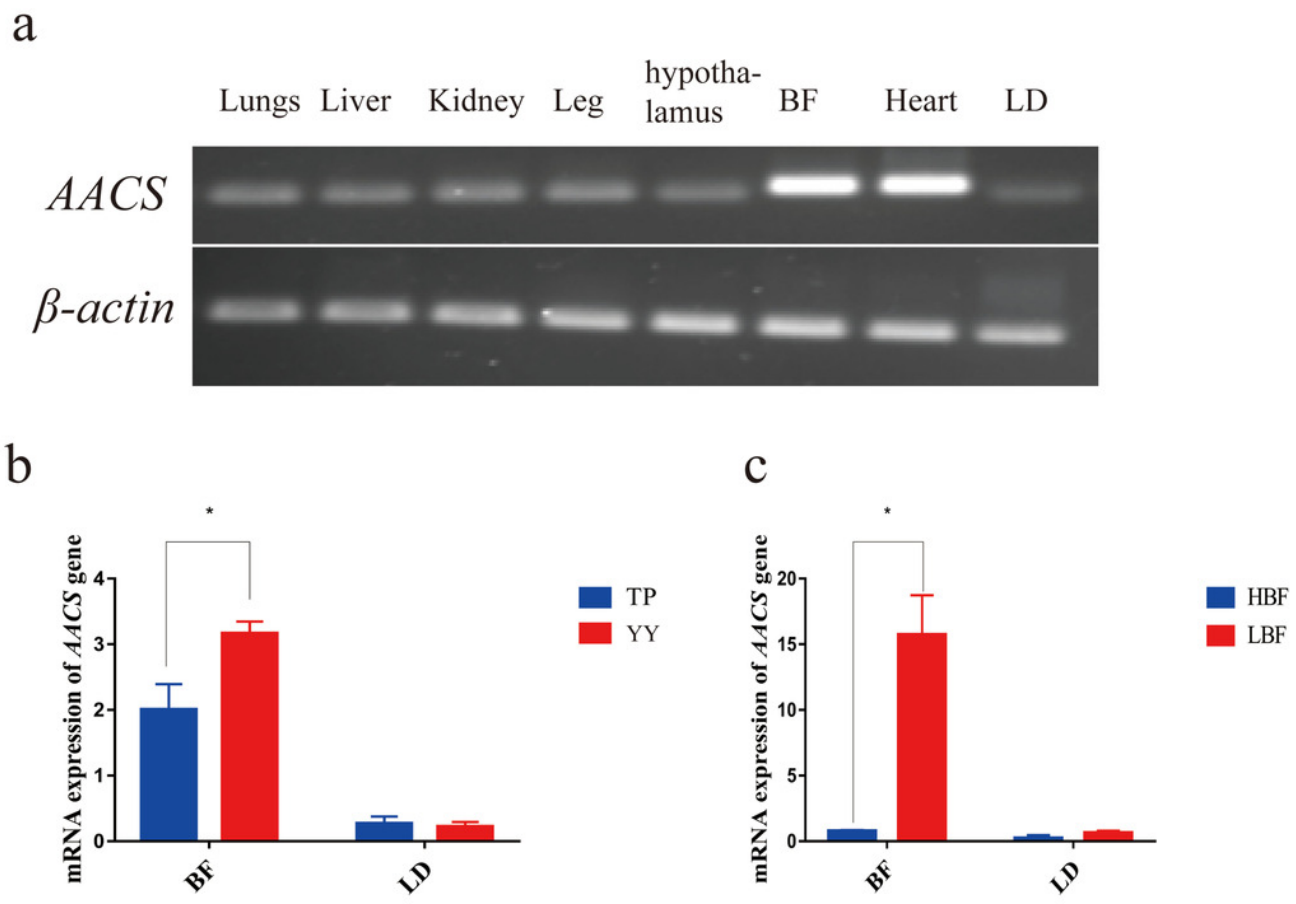
(a) Amplification of 5′ RACE (left), PCR (middle), and 3′ RACE (right). (b) Prediction of hydrophilicity of the AACS protein. (c) Transmembrane domain prediction of the AACS protein. M= T rans2K/ Trans2K Plus DNA Marker(Transgene, Beijing, China).



# Figure 2

Figure 2. Expression analysis of *AACS* in tissues.

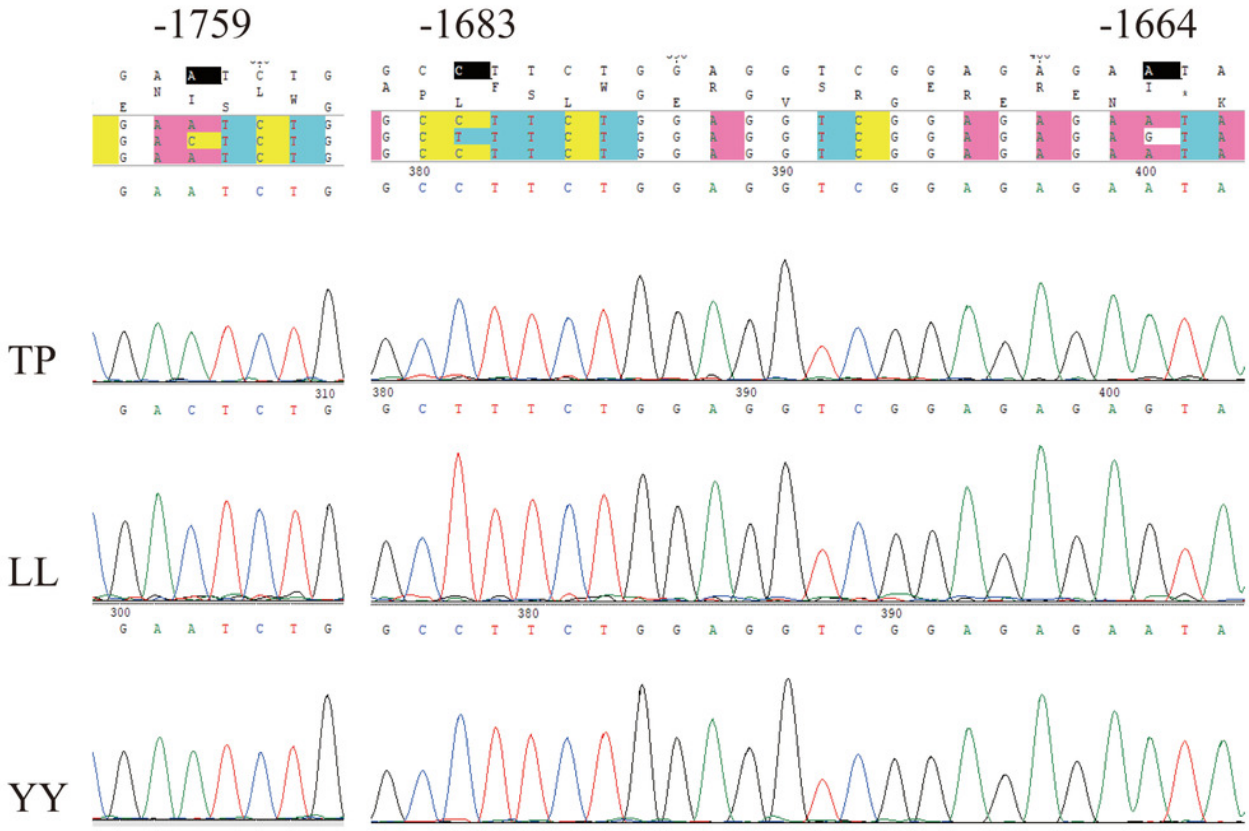
(a) *AACS* expression in different tissues of TP pigs using SqRT-PCR. (b) The mRNA expression of *AACS* in TP and YY pigs. (c) The mRNA expression of *AACS* in DY pigs. BF, back fat; LD, longest dorsal; TP, Tibetan pig; YY, Yorkshire pig; HBF, high back fat thickness; LBF, low back fat thickness. Each bar represents the means ± SD. * $P < 0.05$.



# Figure 3

Figure 3. Sequencing map of three linked sites.

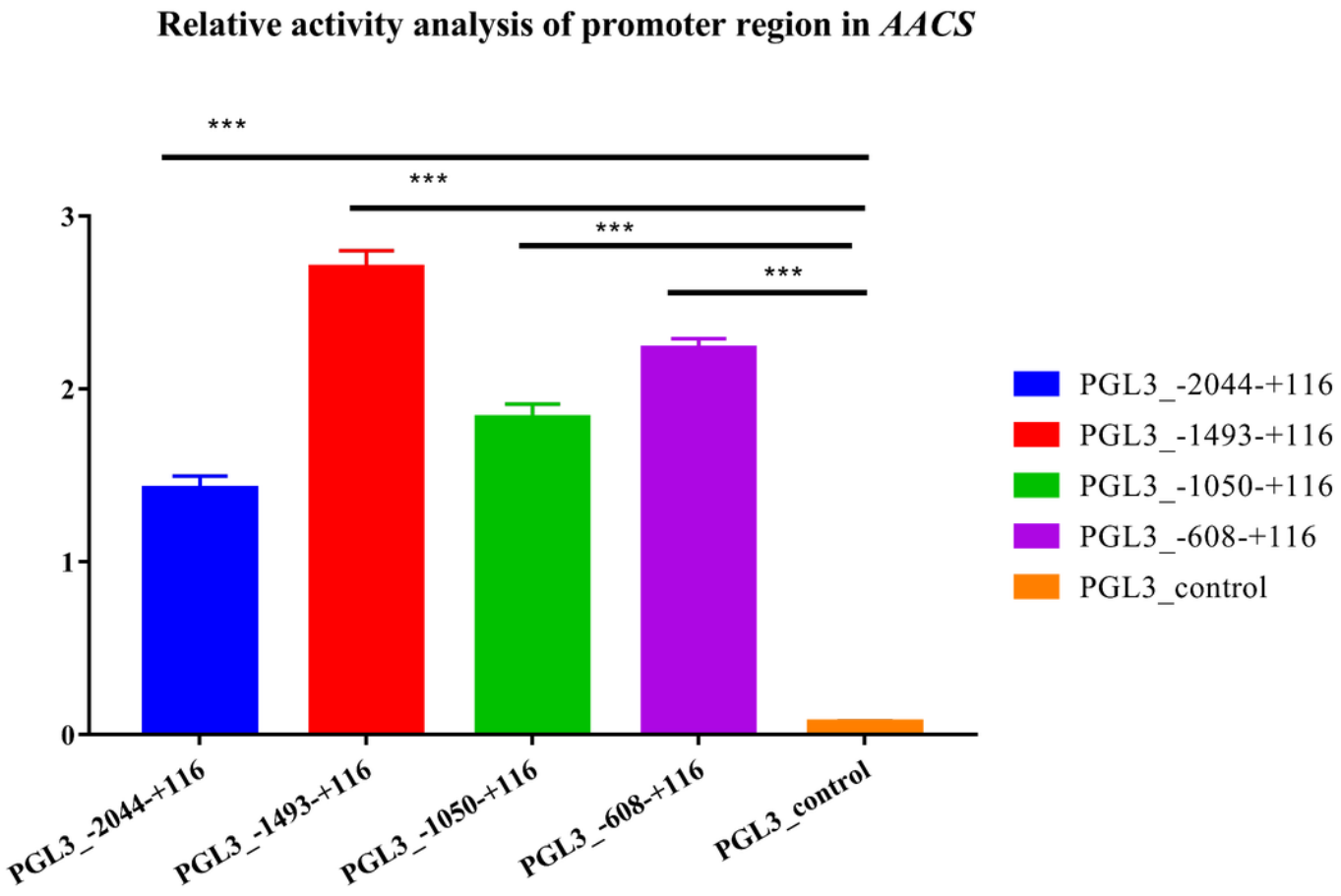
TP, Tibetan pig; YY, Yorkshire; LL, Landrace.



# Figure 4

Figure 4. Dual-luciferase analysis for promoter activity.

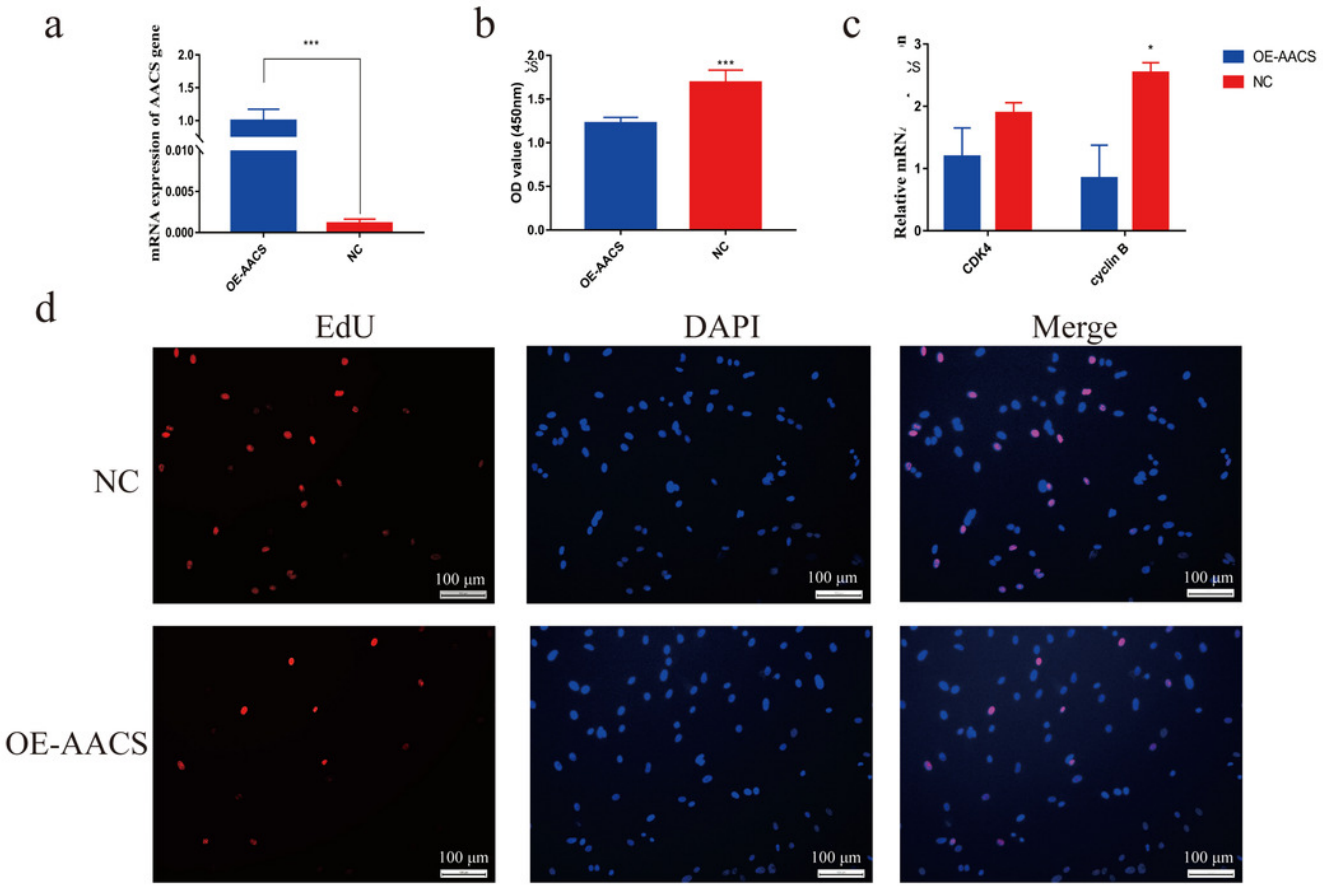
The control group was co-transfected with PGL3-basic and PRL-TK. Each bar represents the means ± SD. *** $P < 0.001$.



# Figure 5

Figure 5. Overexpression of *AACS* inhibits the proliferation of porcine subcutaneous preadipocytes.

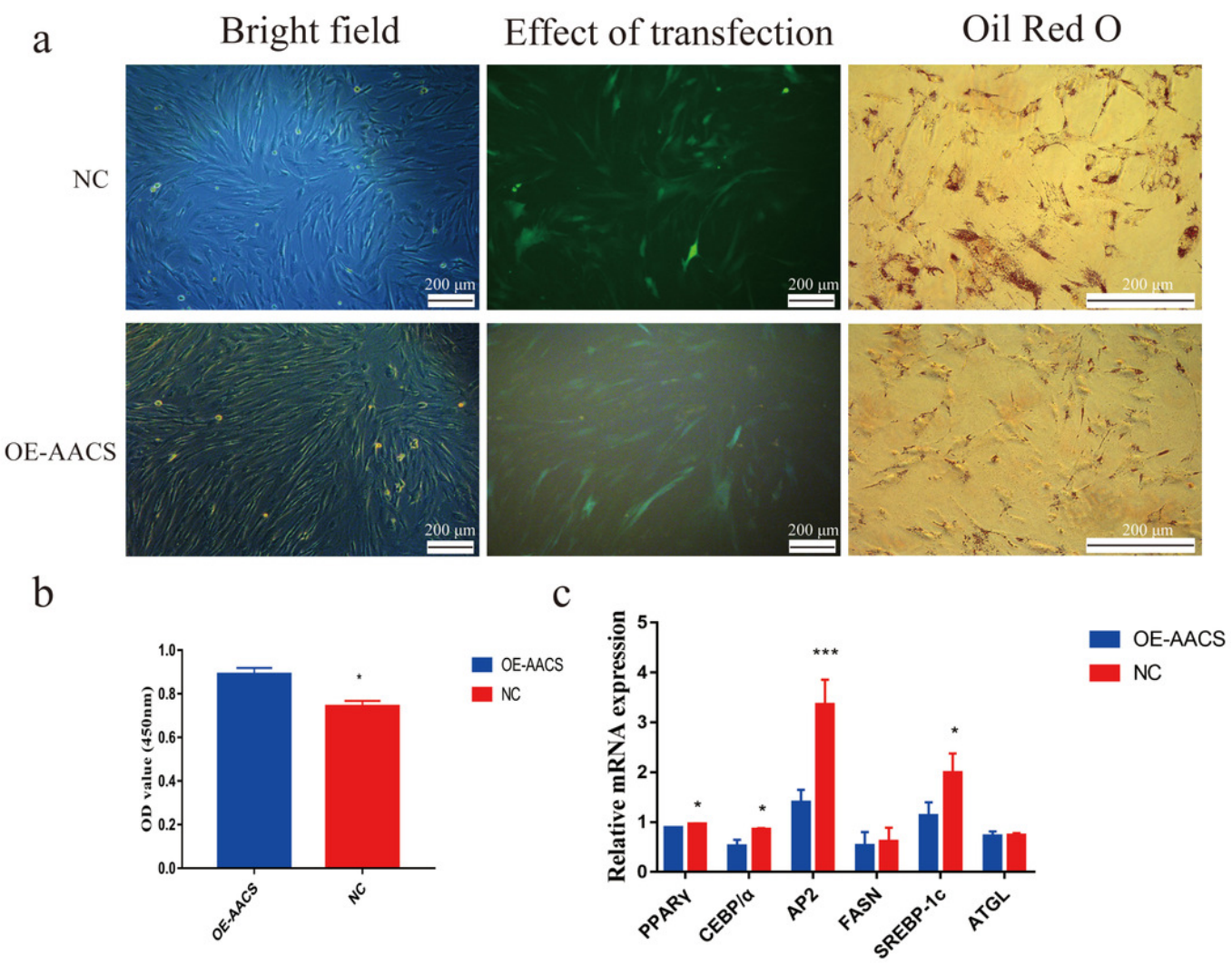
(a) Detection of overexpression efficiency after lentivirus infection. (b) Overexpression of *AACS* inhibited the proliferation of porcine subcutaneous preadipocytes using CCK8 assay. (c) The mRNA expression levels of proliferation-related genes . (d) The proliferation of porcine subcutaneous preadipocytes after overexpression of *AACS* for 24 h was detected using EdU staining. Each bar represents the means ± SD. * $P < 0.05$, *** $P < 0.001$.



# Figure 6

Figure 6. Overexpression of *AACS* inhibited the differentiation of porcine subcutaneous preadipocytes.

(a) Image of porcine adipocytes stained with oil red O after six days of differentiation. (b) Oil-red O staining was used to determine the content of lipid droplets in porcine subcutaneous adipocytes. (c) The mRNA expression of marker genes associated with adipogenesis in porcine subcutaneous adipocytes. Each bar represents the means ± SD. * $P < 0.05$, *** $P < 0.001$.


a
Bright field
Effect of transfection
Oil Red O
NC
OE-AACS
200 μm
b
OD value (450nm)
1.0
0.8
0.6
0.4
0.2
0.0
*
OE-AACS
NC
c
Relative mRNA expression
5
4
3
2
1
0
*
*
***
*
OE-AACS
NC
PPARγ
CEBPIα
AP2
FASN
SREBP-1c
ATGL

## Table 1(on next page)

Table 1. Gene and genotype frequencies of A-1759C sites in different pig breeds.

1 **Table 1.** Gene and genotype frequencies of A-1759C sites in different pig breeds.

| **Breed** | **Genotype frequency（number/frequency）** | | | | **Allele frequency** | |
|---|---|---|---|---|---|---|
| | AA | AC | CC | HWE $\chi^2$ value (*P*-value) | A | C |
| TP | 32/0.9412 | 2/0.0588 | 0/0.0000 | 0.031 (*P* = 0.860) | 0.9706 | 0.0294 |
| LL | 2/0.0588 | 12/0.3529 | 20/0.5882 | 0.013 (*P* = 0.911) | 0.2353 | 0.7647 |
| YY | 6/0.15 | 17/0.425 | 17/0.425 | 0.137 (*P* = 0.934) | 0.3625 | 0.6375 |

## Table 2(on next page)

Table 2. Association analysis of A-1759C with back fat thickness and growth traits

Note: Letters following the data indicate significant differences. Interphase letters indicate significant differences ($P < 0.01$); the same letters indicate non-significant differences ($P > 0.05$).

1 **Table 2.** Association analysis of A-1759C with back fat thickness and growth traits

| Genotype | AA (n = 34) | AC (n = 58) | CC (n = 12) |
|---|---|---|---|
| Thickness of back fat | 14.16 ± 3.17[a] | 14.24 ± 3.28[a] | 10.95 ± 2.11[c] |
| Day old at 70 kg | 216.36 ± 15 | 213.24 ± 16.11 | 214.14 ± 12.32 |

2 Note: Letters following the data indicate significant differences. Interphase letters indicate significant
3 differences ($P < 0.01$); the same letters indicate non-significant differences ($P > 0.05$).
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