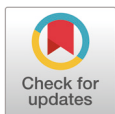


Low lysine stimulates adipogenesis through ZFP423 upregulation in bovine stromal vascular cells

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Abstract

Adipogenesis is a complex process comprising commitment and a differentiation stages. Through research, many different transcriptional factors were found to mediate preadipocyte commitment and differentiation. Lysine has a potential of regulating the commitment and differentiation of preadipocytes. In the present study, intramuscular stromal vascular cells (SVC) isolated from Hanwoo beef cattle were used to elucidate the effects of low lysine level on adipogenesis. SVC were isolated and incubated with various concentrations of lysine (0, 37.5, 75, 150 and 300 µg/mL). No significant difference were observed in the proliferation of SVC after 24 and 48 h of incubation with different concentration of lysine. On preadipocyte determination, reducing the level of lysine significantly increased the expression of preadipocyte commitment gene Zinc finger protein 423 and Preadipocyte factor-1. Upon differentiation, Oil Red O staining revealed that lipid accumulation and triglyceride content significantly increased with the decreasing lysine levels in the media. Expression levels of peroxisome proliferator-activated receptor-γ, CCAAT enhancer binding protein-α, sterol regulatory element binding protein-1c, Fatty Acid Binding Protein 4 and stearoyl CoA desaturase were upregulated by the decreased level of lysine. These data suggest the potential mechanism of action for the improved preadipocyte commitment and adipocyte differentiation in bovine intramuscular SVC upon treatment with low levels of lysine. These findings may be valuable in developing feed rations that promote deposition of intramuscular fat in beef cattle through lysine level modification.

Keywords: Lysine, Stromal vascular cells, Intramuscular fat, Hanwoo beef cattle, Adipogenesis

INTRODUCTION

Intramuscular fat deposition is an important characteristic of meat quality in ruminants because of its positive correlation with meat flavor. The formation of intramuscular fat is a very complex process affected and regulated by multiple factors which are directly or indirectly involved in cell proliferation, differentiation and fatty acid metabolism [1]. Many of the important aspects of fat synthesis in beef cattle occurs at the molecular level. One of the latest targets of nutrigenomic research is the correlation of nutritional factors with the alteration of its metabolism [2].

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Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions

Conceptualization: Hwang SG.
Data curation: dela Cruz JF, Pacunla KWM.
Formal analysis: dela Cruz JF.
Methodology: dela Cruz JF, Hwang SH.
Software: Pacunla KWM.
Validation: Hwang SG.
Investigation: Hwang SG.
Writing - original draft: dela Cruz JF, Pacunla KWM.
Writing - review & editing: dela Cruz JF, Pacunla KWM, Hwang SG.

Ethics approval and consent to participate

This article does not require IRB/IACUC approval because there are no human and animal participants.

In Korea, the conventional feeding stipulates a restricted feeding of concentrates but a steady increase from 50% to 90% in the provided concentrate to forage ratio to amply allocate energy to promote deposition of fat during the 17-month of fattening phase [3]. Forages are usually supplied *ad libitum* during the growing stage (6–11 months) and are slowly decreased from 30% to 10% of total dry matter (DM) intake from the early (12–20 months) to late fattening (21–29 months) stages, respectively [4]. In concentrate feeds, the amount of total digestible nutrient (TDN) can be gradually increased to up to 74% DM, and crude protein (CP) can be reduced from 16% to 12% DM [5]. However, some commercial farms have been modifying their fattening stage by supplying concentrate feeds with a CP content higher than that in conventional feeds during the entire fattening stage. These different practices that changes the supplied CP in the diet might affect the amino acid profile of the feed and affect the mechanism of fat deposition in Hanwoo beef cattle.

Amino acids are building blocks of proteins and alternative sources of energy. They are essential for various physiological functions, which includes antioxidant activity, stress reducing properties, immunomodulation and indirect improvement in meat quality [6]. However, recent researches have also recognized the function of amino acids as signal transducers in major metabolic pathways [7,8]. Lysine is a promising amino acid. Although reports are inconsistent, amino acids are reported to induce lipid accumulation *in vivo* and *in vitro* [9,10]. The mechanisms involved in enhancing adipocyte differentiation through decreasing level of lysine are still not fully understood. One of the potential pathways that can lead to increased adipogenesis is through increased preadipocyte commitment via Zinc finger protein 423 (Zfp423). Zfp423 was recently identified as a critical marker of committed preadipocytes and a key initiator of adipocyte differentiation [11]. We hypothesized that low lysine levels may differentially regulate the Zfp423 signaling pathway to increase preadipocyte commitment of bovine stromal vascular cells (SVC) resulting in increased adipocyte differentiation.

MATERIALS AND METHODS

Isolation of bovine stromal vascular cells

Bovine intramuscular adipose tissue was collected directly at the cervical longissimus dorsi muscle of Hanwoo beef cattle from Dodram Slaughterhouse, Anseong, Korea. The samples were immediately delivered to the Applied Biochemistry Laboratory at Hankyong National University in a sterile phosphate-buffered saline (PBS) supplemented with Amphotericin B (2 ng/mL) and gentamycin (0.2 mg/mL). The dissected adipose tissues were then finely minced and incubated in Dulbecco's Modified Essential Medium (DMEM) containing collagenase type 1 (2 mg/mL) and bovine serum albumin (4 mg/mL). Digestion was performed for 50 min in a water bath set at 37°C with gentle shaking. After digestion, 5 mL of DMEM were added to stop the digestion process. The digested adipose tissues were then filtered through a 100- μ m nylon cell strainer, and the suspension was centrifuged at 700 \times g for 10 min. The supernatant and lipid layer were removed by suction. The collected pellet was washed three times with DMEM. The cells were resuspended and incubated in the growing media composed of DMEM with 10% bovine calf serum (BCS), 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C under a humidified atmosphere and 5% CO₂.

Cell proliferation assay

To determine the effect of different lysine concentration on the proliferation of isolated SVC, a cell proliferation assay was performed using Cell Counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) following the manufacturer's instruction. Briefly, the cells were seeded at a density of 1×10^4 cells per well and grown in DMEM BCS media supplemented with increasing levels of lysine (0

to 300 µg/mL) for 24 and 48 h. After incubation, the media were removed and replaced with new DMEM with 10 µL of CCK-8 reagent was added per well. The plates were then incubated at 37°C for 2h. Absorbance at 450 nm was measured using an ELISA plate reader (Tecan, Switzerland).

Differentiation of bovine stromal vascular cells

Bovine SVC were cultured in DMEM supplemented with 10% BCS until they reached 80% to 90% confluence. Differentiation media with different concentrations of lysine and containing 10% fetal bovine serum (FBS), 1 µM dexamethasone (DEX, Sigma-Aldrich, St. Louis, MO, USA), 0.5 mM isobutyl methylxanthine (IBMX, Sigma-Aldrich), and 10 µg/mL insulin (INS, Sigma-Aldrich) were used. After 2 days, the media was replaced by DMEM supplemented with 10% FBS and 10 µg/mL insulin. The cells were maintained in DMEM supplemented with FBS until day 10.

Morphological analysis

Oil Red-O (ORO) staining was used to determine the extent of adipocyte differentiation at day 10. Differentiated bovine SVC were fixed in 10% formalin for 1 hour, washed with distilled water, and stained with 0.6% Oil Red-O solution (Sigma Aldrich) in the dark for 15 min. After staining, the cells were washed with water and coated with glycerol for imaging. Differentiation was observed by microscopy, relative lipid content was measured by isopropanol elution and optical density was evaluated at 520 nm.

RNA isolation and reverse transcriptase polymerase chain reaction

To determine preadipocyte commitment, bovine SVC were grown in six-well plates with DMEM containing 10% BCS and different concentration of lysine (0, 37.5, 75, 150 and 300 µg/mL) for 48 h. After 48 h, total RNA was isolated from the bovine SVC using RNAiso Plus (TaKaRa Bio, Kusats, Japan). The concentration and purity of RNA was determined at an absorbance of 260 nm and 280 nm, respectively using a Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription was conducted with Maxime RT Premix Kit (iNtRON Biotechnology, Seongnam, Korea) following the manufacturer's recommendations.

For the evaluation of adipocyte differentiation, total RNA was isolated using the previously described procedure using bovine SVC differentiated for 10 days. PCR consisted of 5 min denaturation process at 95°C, gene amplification for 32 cycles: 40s at 95°C, primer attachment for 40 seconds (annealing temperature 56°C–68°C), and extension at 72°C for 1 min. The following oligonucleotide primers were used in RT-PCR: Zfp423 forward: GGA TTC CTC CGT GAC AGC A; Zfp423 reverse: TCG TCC TCA TTC CTC TCC TCT; Pref1 forward: AAG CAC CGG CAG ACA AGA; Pref1 reverse: CAG AGG AAG GAG TCG TCA GTA; peroxisome proliferator-activated receptor-gamma (PPAR γ) forward: CCG CTT CCA GGG GTG TCA GT; PPAR γ reverse: GGA TAT GAG GAC CCA TCC T; CCAAT enhancer binding protein alpha (C/EBP α) forward: ACT AAC CAG TGA CAA TGA CC; C/EBP α reverse: CTT GAC CAG GGA GCT CTC G; sterol regulatory element binding transcription factor 1c (SREBP-1c) forward: GAC GCT CTT ACA TCA ATG AC; SREBP-1c reverse: TTC GGC CAT TTG CTT TTG TG; fatty acid binding protein 4 (FABP4) forward: ACT TAG ATG AAG GTG CTC TG ; FABP4 reverse; CCT CAG GAC TAA ACA ACT TAT G; stearyl-CoA desaturase (SCD) forward: CCT GTG GAG TCA CCG AAC C; SCD reverse: CCT TGG ATA CTT TCT TCC GGT C; and β -actin forward: GGC CAT GGA TGA TGA TAT TGC; β -actin reverse: ACG CCG CCT TTC ACA T.

Western blot analysis

SVC were grown in six-well plates and was allowed to differentiate for 10 days with different levels of lysine. Protein extraction was performed on day 10 by adding protein extraction solution (iNtRON Biotechnology). The protein samples were purified by centrifugation at 15,000 ×g for 15min at 4°C and the protein content of the supernatant was measured using a modified Bradford assay. Prepared protein samples were allowed to separate by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blocking was performed with 5% skimmed milk and the samples were incubated with the following primary antibodies (Cell Signaling Technology, Danvers, MA, USA): rabbit polyclonal anti-PPAR γ , rabbit polyclonal anti-C/EBP α , polyclonal anti-SREBP-1c, rabbit polyclonal anti-FABP4, mouse monoclonal anti-SCD, and mouse monoclonal anti- β -actin. Proteins were detected by incubating the nitrocellulose membranes with secondary antibodies conjugated with horseradish peroxidase followed by a treatment with enhanced chemiluminescence (AB Frontier, Seoul, Korea). The specific proteins of concern were evaluated using radiographic film exposure.

Statistical analyses

All the data are presented as mean \pm SD. Statistical differences between the control and treatment group for cell proliferation, relative lipid accumulation, and mRNA and protein expression were analyzed by one-way analysis of variance (ANOVA) with Turkey's honestly significant difference (HSD) post-hoc test (SPSS v 23). Values were considered statistically significant at $p < 0.05$. All presented data are representative of at least three independent tests ($n \geq 3$).

RESULTS AND DISCUSSION

Intramuscular fat deposition is one of the most important qualities of meat and can translate into a massive economic benefit for the beef industry. Specific feeding strategies have been developed in Korea to improve the quality of Hanwoo beef. Changes in the CP content of the diet have been the focus of several studies that aimed to promote intramuscular fat deposition but which yielded conflicting results [3,12,]. The effects of dietary protein and amino acid levels on the transcriptional expression of genes responsible for fatty acid biosynthesis during the fattening stage of Hanwoo beef cattle remain unclear. However, recent studies have shown that fibroblasts and adipocytes originate from common progenitor cells that are mainly located in the stromal vascular fraction of the skeletal muscles [13–15]. SVC isolated from skeletal tissues are composed of a diverse population of cells. These cells can easily be grown *in vitro* and with the proper stimulation, have great potential to differentiate into different cell lineages. In beef cattle, the regulation of the mechanisms involved in the early commitment of intramuscular SVC to the adipogenic lineage are still unclear. In this study, we demonstrated that decreasing the level of lysine in the media increased the preadipocyte commitment, differentiation and lipid accumulation in Hanwoo intramuscular SVC.

To determine the potential impact of lysine on the viability of SVC from Hanwoo beef cattle, isolated SVC were grown in DMEM BCS media containing 0 to 300 μ g/mL of lysine. The effect of lysine levels on the proliferation of SVC is shown in Fig. 1. The lysine concentration of up to 300 μ g/mL had no significant effect on the proliferation of Hanwoo SVC. Thus, lowering or increasing the lysine level had no detrimental effect on the proliferation of Hanwoo SVC.

The effect of lysine levels on preadipocyte determination was evaluated. A lower level of lysine in the media resulted in significantly higher expression of Zfp423 and Pref-1 in Hanwoo SVC after 24 and 48 h of incubation (Fig. 2). This finding indicates that preadipocyte commitment is

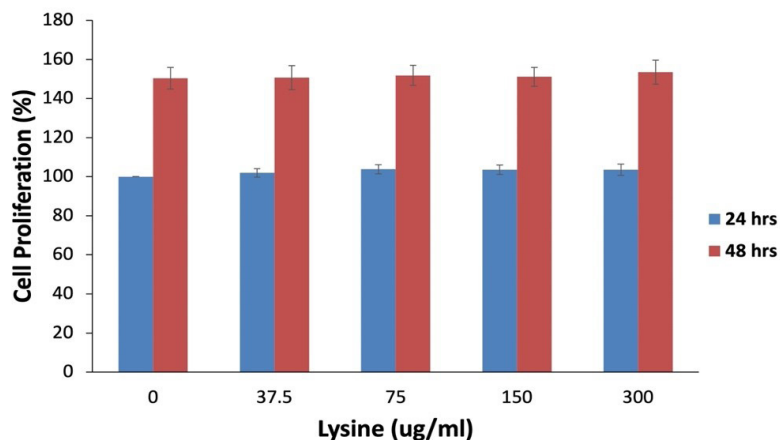


Fig. 1. Effect of lysine concentration on the proliferation of Hanwoo SVC incubated for 24 and 48 hrs. Reported values are means \pm SD. SVC, stromal vascular cells.

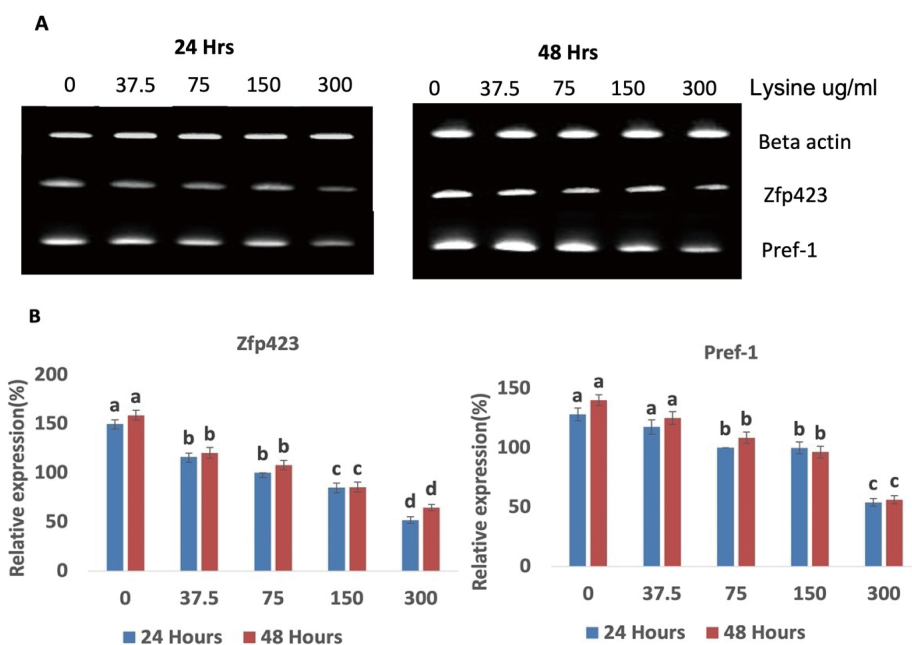


Fig. 2. Effect of lysine concentration on the mRNA expression of Zfp423 and Pref-1 gene after 24 and 48 hours. (A) Representative RT-PCR bands. (B) Relative mRNA expression (%). Reported values (mean \pm SD) are the result of three independent experiments. Bars annotated with different letters are significantly different ($p < 0.05$). RT-PCR, reverse transcriptase polymerase chain reaction.

negatively correlated with lysine levels. Zinc finger protein 423 (Zfp423) was recently identified as a key transcriptional factor that induces PPAR γ expression and is crucial for the early commitment of progenitor cells to adipogenesis [11]. Preadipocyte factor-1 (Pref-1) is highly expressed in preadipocytes but decreases during differentiation and is absent in mature adipocytes. Thus, it is used as a common preadipocyte marker [16–18]. Our results showed that exposure of intramuscular SVC to different concentrations of lysine significantly affected the expression of these two transcriptional markers. Decreasing the concentration of lysine in the growth media significantly increased the expression of the pre-adipocyte commitment marker Zfp423 and preadipocyte

determination marker Pref-1 (Figs. 2A and 2B). These data suggests that low lysine levels resulted in increased commitment of intramuscular SVC to the adipogenic lineage by the upregulation of Zfp423. This was confirmed by the increased expression of Pref-1 which is highly expressed in preadipocytes. The findings agree with those of Jing et al. [19], who observed a transient rise in Pref-1 levels in mesenchymal stem cells during the commitment stage. Correlating the adipocyte determination and proliferation data indicates that although lysine levels did not significantly affect the proliferation of Hanwoo SVC, the preadipocyte determination data showed that lower levels of lysine significantly increased the commitment of Hanwoo SVC to the adipogenic lineage, which can potentially be stimulated to become mature adipocytes.

We performed a 10-day differentiation induction experiment in Hanwoo beef cattle SVC cultured in DMEM media with increasing levels of lysine (0, 37.5, 75, 150 and 300 $\mu\text{g}/\text{mL}$) and the level of intracellular lipid concentration was determined using Oil Red-O Staining. As the level of lysine in the differentiation media decreased, the intracellular lipid accumulation is significantly increased (Figs. 3A and 3B). After 10 days of differentiation, Hanwoo SVC grown without lysine significantly accumulated lipid droplets (Fig. 3A). Quantification of relative lipid accumulation obtained by measuring the absorbance at 520 nm after the extraction of the triglycerides stained with Oil Red-O provided confirmation (Fig. 3B).

Several studies have previously evaluated the potential effects of lysine on the regulation of lipid metabolism in animals. Goda et al. [9] reported that triglycerides accumulate more in the muscle of rats fed with dietary lysine restriction. Katsuma et al. [20] conveyed that a lysine-deficient diet can increase the lipid content in the skeletal muscle of pigs. Beloor et al. [10] also showed that insufficient supply of lysine causes an increase in the adipogenic potential of bovine preadipocytes. These studies highlight the possibility that lysine plays a significant role in the control of lipid metabolism. Our differentiation data mirrored the claim of these researchers that lysine restriction increases triglyceride accumulation. Oil Red-O staining classifies adipocytes from other cells and has been used as a method to quantify and assess changes in the degree of adipogenesis in cell culture [21]. Oil Red-O staining revealed that a decrease in lysine concentration resulted in increased adipocyte differentiation and relative lipid content (Figs. 3A and 3B). These data can be attributed to the higher preadipocyte determination in Hanwoo SVC grown with low levels of lysine. We observed significantly showed that increased expression of Zfp423 and Pref-1 which are excellent markers for preadipocyte determination. More pronounced preadipocyte commitment due to Zfp423 and Pref-1 upregulation then resulted in an increased level of adipocyte differentiation and relative lipid content which was confirmed by our Oil Red-O data.

To determine the effect of lysine on the possible intracellular mechanisms regulating the differentiation of Hanwoo SVC, the expression of major adipogenic transcription factors during differentiation was monitored using RT-PCR and Western Blot analysis. The results of RT-PCR and western blot analyses are summarized in Figs. 4A and 4B, and Figs. 5A and 5B, respectively. In the study of Huang et al. [15] and Gupta et al. [11], they showed that Zfp423 is a critical regulator of adipogenesis in bovine SV cells and that upregulation of Zfp423 intensifies the expression of PPAR γ and C/EBP α . This pattern was also observed in this study. As the lysine concentration decreased, the expression of Zfp423 in intramuscular SVC increased and in return upregulated the expressions of PPAR γ and C/EBP α . Multiple studies have shown that PPAR γ and C/EBP α are considered to be the major transcription factors that facilitate adipogenesis [22–24]. PPAR γ stimulates and promotes C/EBP α expression and vice versa, which creates a positive-feedback reaction. Additionally, PPAR γ and C/EBP α also stimulate the expression of genes that are involved in lipogenesis, lipolysis and insulin sensitivity [25,26]. FABP4 and SREBP-1c, which stimulates lipid accumulation in cells are both regulated by PPAR γ and C/EBP α [27]. We observed that

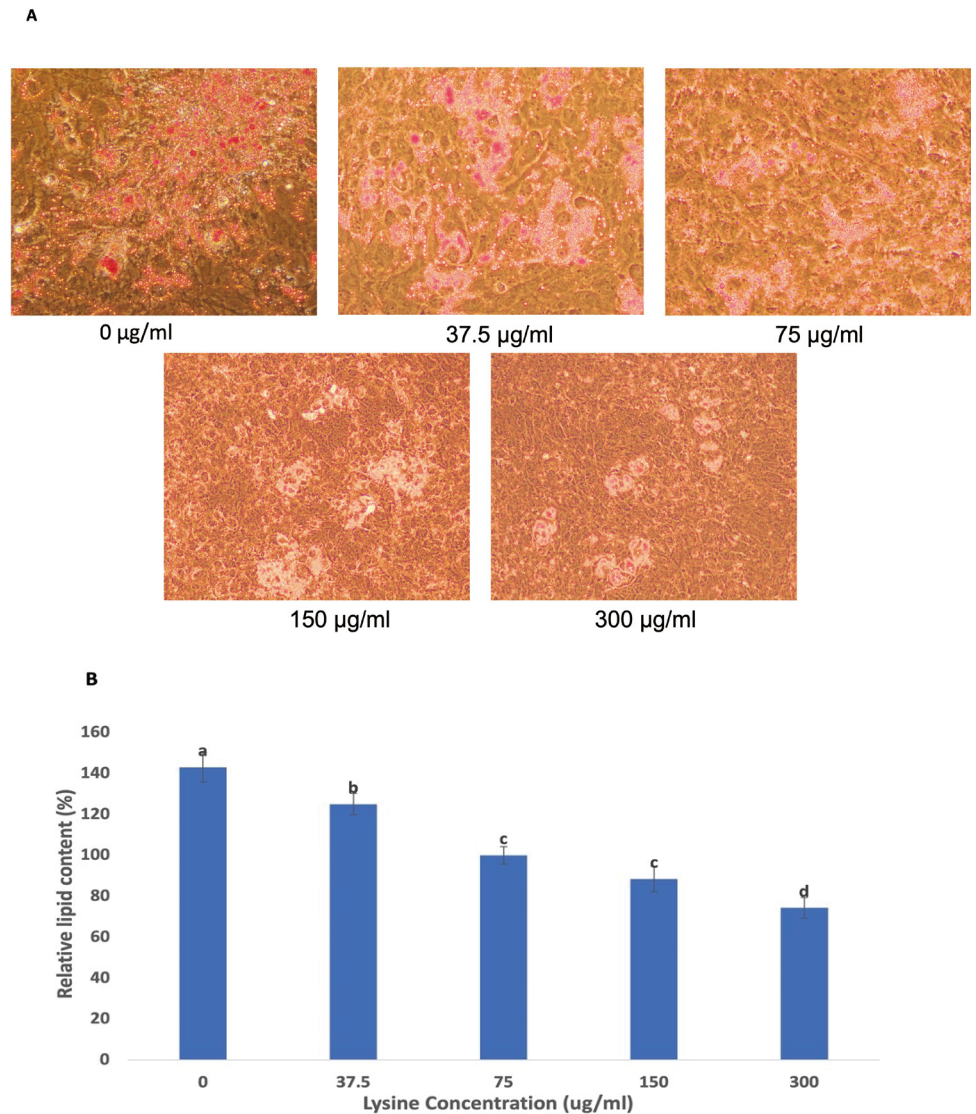


Fig. 3. Effect of lysine concentration on the differentiation and lipid accumulation in Hanwoo SVC. (A) Representative microscopic morphological images of adipocytes stained with Oil Red-O after 10 days of differentiation. (B) Relative lipid content by quantification method of Oil Red-O staining. Values are expressed as mean \pm SD of three independent experiments. Bars annotated with different letters are significantly different ($p < 0.05$). SVC, stromal vascular cells.

lowering the lysine level in the media significantly upregulated FABP4 and SREBP-1c expression.

The SCD1 gene has been identified as the major limiting enzyme in the synthesis of monounsaturated fatty acids (MUFAs) and is transcriptionally regulated by several factors [28]. SCD1 reportedly contributes to the improvement of beef quality by improving the fatty acid composition [29]. Through the action of the SREBP-1c, a lipogenic transcription factor, insulin and glucose mediated stimulation of SCD1 occurs [28,30]. Our data clearly shows that lysine levels significantly affected the expression of SREBP-1c and in return also altered SCD1 expression. This upregulation of SCD1 expression may prove useful to improve the flavor of meat with a healthier fatty acid content.

In summary, our data establish the significant role of Zfp423 in the regulation of adipogenesis in Hanwoo intramuscular SVC. Zfp423 increases the commitment of SVC to the adipogenic lineage

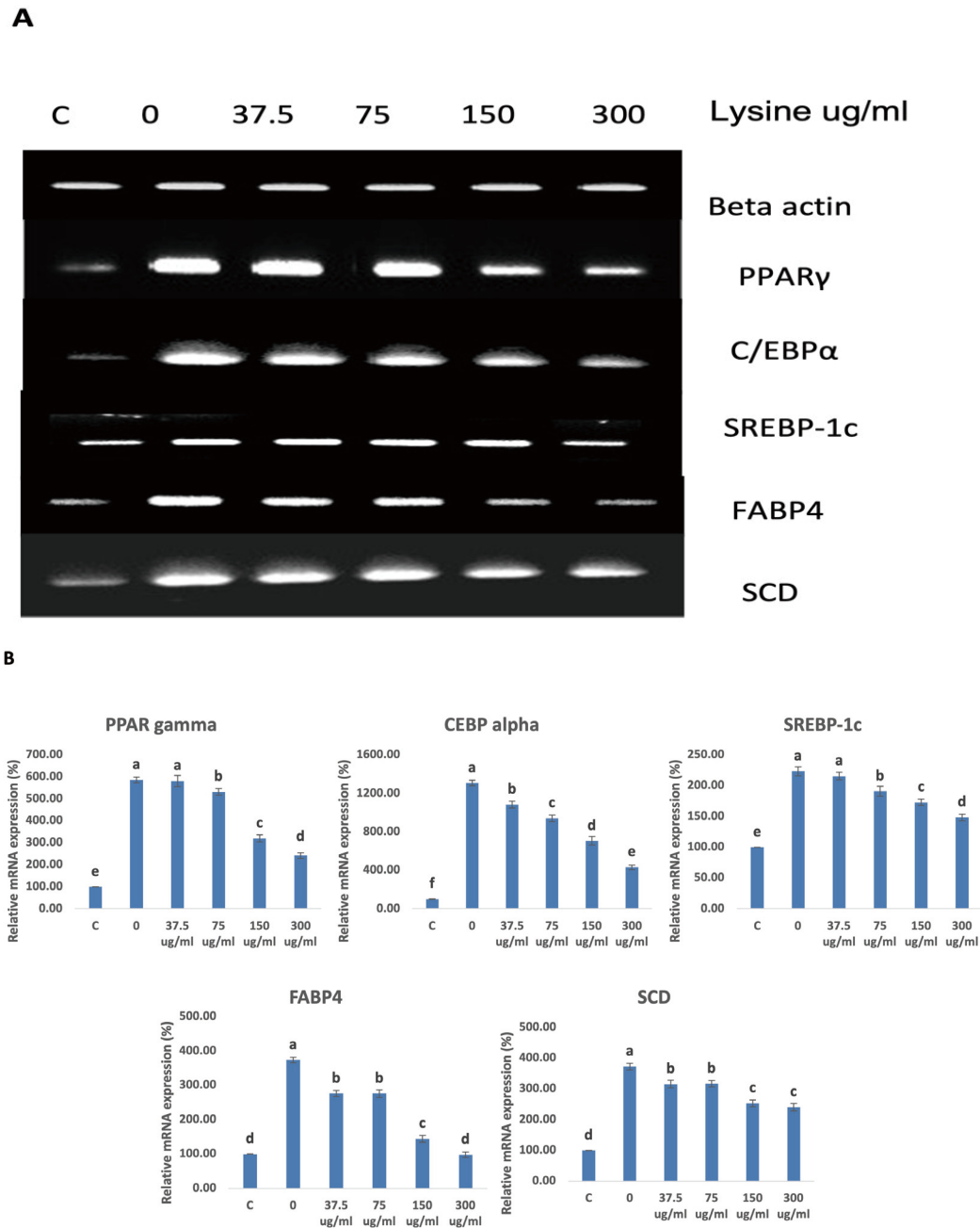


Fig. 4. Effect of lysine concentration on the mRNA expression of adipogenic transcription factors. (A) Representative RT-PCR bands. (B) Relative mRNA expression (%). Reported values (mean \pm SD) are the result of three independent experiments. Group C is negative control without differentiation cocktail. Bars annotated with different letters are significantly different ($p < 0.05$). PPAR γ , peroxisome proliferator-activated receptor-gamma; C/EBP α , CCAAT enhancer binding protein alpha; SREBP-1c, sterol regulatory element binding transcription factor 1c; FABP4, fatty acid binding protein 4; SCD, stearoyl-CoA desaturase; RT-PCR, reverse transcriptase polymerase chain reaction.

while enhancing PPAR γ and C/EBP α expression. This cascade of events results in the upregulation of important adipogenic genes, FABP4, SREBP-1c and SCD1. Oil Red-O staining clearly showed enhanced adipogenic differentiation in lysine deficient SVC. These data suggests that low lysine levels upregulated Zfp423 expression and resulted in an increased preadipocyte determination and enhanced intramuscular adipogenesis in Hanwoo SVC.

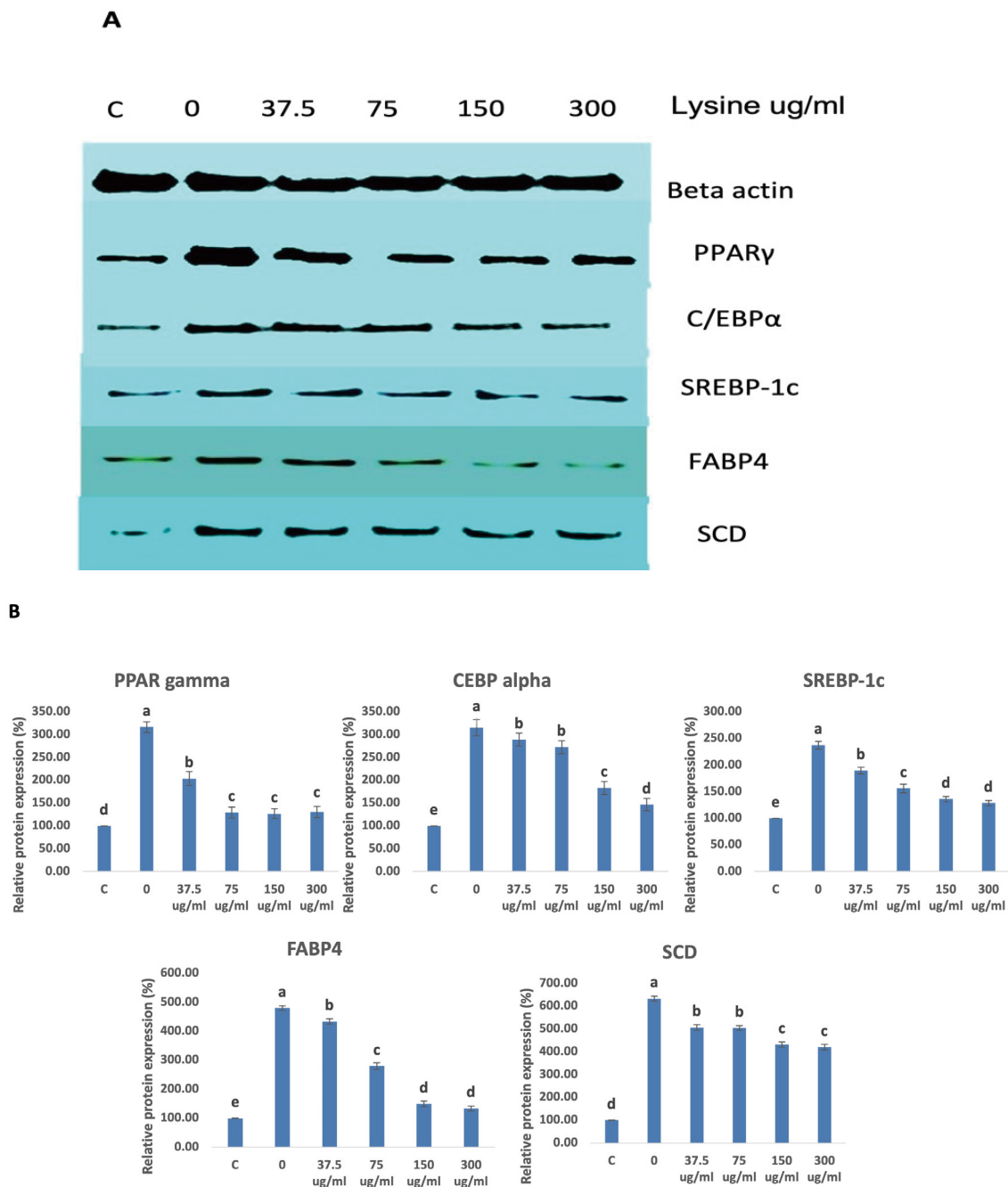


Fig. 5. Effect of lysine concentration on the protein expression of adipogenic transcription factors. (A) Representative Western blot bands. (B) Relative protein expression (%). Reported values (mean \pm SD) are the result of three independent experiments. Group C is negative control without differentiation cocktail. Bars annotated with different letters are significantly different ($p < 0.05$). PPAR γ , peroxisome proliferator-activated receptor-gamma; C/EBP α , CCAAT enhancer binding protein alpha; SREBP-1c, sterol regulatory element binding transcription factor 1c; FABP4, fatty acid binding protein 4; SCD, stearoyl-CoA desaturase.

REFERENCES

1. Malgwi IH, Halas V, Grünvald P, Schiavon S, Jócsák I. Genes related to fat metabolism in pigs and intramuscular fat content of pork: a focus on nutrigenetics and nutrigenomics. *Animals*.

- 2022;12:150. <https://doi.org/10.3390/ani12020150>
2. Ladeira MM, Schoonmaker JP, Gionbelli MP, Dias JCO, Gionbelli TRS, Carvalho JRR, et al. Nutrigenomics and beef quality: a review about lipogenesis. *Int J Mol Sci.* 2016;17:918. <https://doi.org/10.3390/ijms17060918>
 3. Bharanidharan R, Thirugnanasambantham K, Ibidhi R, Bang G, Jang SS, Baek YC, et al. Effects of dietary protein concentration on lipid metabolism gene expression and fatty acid composition in 18–23-month-old Hanwoo steers. *Animals.* 2021;11:3378. <https://doi.org/10.3390/ani11123378>
 4. National Institute of Animal Science. 2012 Annual report. Suwon: National Institute of Animal Science; 2013. Report No.: 11-1390906-000217-10.
 5. Jo C, Cho SH, Chang J, Nam KC. Keys to production and processing of Hanwoo beef: a perspective of tradition and science. *Anim Front.* 2012;2:32-8. <https://doi.org/10.2527/af.2012-0060>
 6. Ma X, Yu M, Liu Z, Deng D, Cui Y, Tian Z, et al. Effect of amino acids and their derivatives on meat quality of finishing pigs. *J Food Sci Technol.* 2020;57:404-12. <https://doi.org/10.1007/s13197-019-04077-x>
 7. Wolfson RL, Chantranupong L, Saxton RA, Shen K, Scaria SM, Cantor JR, et al. Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science.* 2016;351:43-8. <https://doi.org/10.1126/science.aab2674>
 8. Saxton RA, Chantranupong L, Knockenhauer KE, Schwartz TU, Sabatini DM. Mechanism of arginine sensing by CASTOR1 upstream of mTORC1. *Nature.* 2016;536:229-33. <https://doi.org/10.1038/nature19079>
 9. Goda Y, Yamanaka D, Nishi H, Masuda M, Kamei H, Kumano M, et al. Dietary lysine restriction induces lipid accumulation in skeletal muscle through an increase in serum threonine levels in rats. *J Biol Chem.* 2021;297:101179. <https://doi.org/10.1016/j.jbc.2021.101179>
 10. Beloor J, Kang HK, Yun CH, Kim SH, Moon YS. Low lysine treatment increases adipogenic potential of bovine intramuscular adipocytes. *Asian-Australas J Anim Sci.* 2009;22:721-6. <https://doi.org/10.5713/ajas.2009.90048>
 11. Gupta RK, Arany Z, Seale P, Mepani RJ, Ye L, Conroe HM, et al. Transcriptional control of preadipocyte determination by Zfp423. *Nature.* 2010;464:619-23. <https://doi.org/10.1038/nature08816>
 12. Kim KH, Oh YG, Lee SC, Shin KJ, Chung WT, Kang SW, et al. Determination of net energy and protein requirements for growth in Hanwoo steers by comparative slaughter experiment. *J Anim Sci Technol.* 2007;49:41-50. <https://doi.org/10.5187/JAST.2007.49.1.041>
 13. Uezumi A, Fukada S, Yamamoto N, Takeda S, Tsuchida K. Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. *Nat Cell Biol.* 2010;12:143-52. <https://doi.org/10.1038/ncb2014>
 14. Uezumi A, Ito T, Morikawa D, Shimizu N, Yoneda T, Segawa M, et al. Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle. *J Cell Sci.* 2011;124:3654-64. <https://doi.org/10.1242/jcs.086629>
 15. Huang Y, Das AK, Yang QY, Zhu MJ, Du M. Zfp423 promotes adipogenic differentiation of bovine stromal vascular cells. *PLOS ONE.* 2012;7:e47496. <https://doi.org/10.1371/journal.pone.0047496>
 16. Sul HS, Smas C, Mei B, Zhou L. Function of pref-1 as an inhibitor of adipocyte differentiation. *Int J Obes.* 2000;24:S15-9. <https://doi.org/10.1038/sj.ijo.0801494>
 17. Sul HS. Minireview: Pref-1: role in adipogenesis and mesenchymal cell fate. *Mol Endocrinol.* 2009;23:1717-25. <https://doi.org/10.1210/me.2009-0160>

18. Wang Y, Kim KA, Kim JH, Sul HS. Pref-1, a preadipocyte secreted factor that inhibits adipogenesis. *J Nutr.* 2006;136:2953-6. <https://doi.org/10.1093/jn/136.12.2953>
19. Jing K, Heo JY, Song KS, Seo KS, Park JH, Kim JS, et al. Expression regulation and function of pref-1 during adipogenesis of human mesenchymal stem cells (MSCs). *Biochim Biophys Acta Mol Cell Biol Lipids.* 2009;1791:816-26. <https://doi.org/10.1016/j.bbalip.2009.04.010>
20. Katsumata M, Kobayashi S, Matsumoto M, Tsuneishi E, Kaji Y. Reduced intake of dietary lysine promotes accumulation of intramuscular fat in the Longissimus dorsi muscles of finishing gilts. *Anim Sci J.* 2005;76:237-44. <https://doi.org/10.1111/j.1740-0929.2005.00261.x>
21. Kraus NA, Ehebauer F, Zapp B, Rudolphi B, Kraus BJ, Kraus D. Quantitative assessment of adipocyte differentiation in cell culture. *Adipocyte.* 2016;5:351-8. <https://doi.org/10.1080/21623945.2016.1240137>
22. Cho YC, Jefcoate CR. PPAR γ 1 synthesis and adipogenesis in C3H10T1/2 cells depends on S-phase progression, but does not require mitotic clonal expansion. *J Cell Biochem.* 2004;91:336-53. <https://doi.org/10.1002/jcb.10743>
23. Giri S, Rattan R, Haq E, Khan M, Yasmin R, Won J, et al. AICAR inhibits adipocyte differentiation in 3T3L1 and restores metabolic alterations in diet-induced obesity mice model. *Nutr Metab.* 2006;3:31. <https://doi.org/10.1186/1743-7075-3-31>
24. Fernyhough ME, Okine E, Hausman G, Vierck JL, Dodson MV. PPAR γ and GLUT-4 expression as developmental regulators/markers for preadipocyte differentiation into an adipocyte. *Domest Anim Endocrinol.* 2007;33:367-78. <https://doi.org/10.1016/j.domaniend.2007.05.001>
25. Payne VA, Au WS, Lowe CE, Rahman SM, Friedman JE, O'Rahilly S, et al. C/EBP transcription factors regulate SREBP1c gene expression during adipogenesis. *Biochem J.* 2009;425:215-23. <https://doi.org/10.1042/BJ20091112>
26. White UA, Stephens JM. Transcriptional factors that promote formation of white adipose tissue. *Mol Cell Endocrinol.* 2010;318:10-4. <https://doi.org/10.1016/j.mce.2009.08.023>
27. Fajas L, Schoonjans K, Gelman L, Kim JB, Najib J, Martin G, et al. Regulation of peroxisome proliferator-activated receptor γ expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism. *Mol Cell Biol.* 1999;19:5495-503. <https://doi.org/10.1128/MCB.19.8.5495>
28. Ntambi JM, Miyazaki M. Regulation of stearyl-CoA desaturases and role in metabolism. *Prog Lipid Res.* 2004;43:91-104. [https://doi.org/10.1016/S0163-7827\(03\)00039-0](https://doi.org/10.1016/S0163-7827(03)00039-0)
29. Ohsaki H, Tanaka A, Hoashi S, Sasazaki S, Oyama K, Taniguchi M, et al. Effect of SCD and SREBP genotypes on fatty acid composition in adipose tissue of Japanese Black cattle herds. *Anim Sci J.* 2009;80:225-32. <https://doi.org/10.1111/j.1740-0929.2009.00638.x>
30. Houdali B, Wahl HG, Kresi M, Nguyen V, Haap M, Machicao F, et al. Glucose oversupply increases Δ 9-desaturase expression and its metabolites in rat skeletal muscle. *Diabetologia.* 2003;46:203-12. <https://doi.org/10.1007/s00125-002-1015-2>