

# Low extracellular calcium and retinoic acid concentration promotes adipocyte differentiation in 3T3-L1 preadipocytes

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**I**ntensive genetic selection of animals for their lean growth has dramatically reduced intramuscular fat (marbling) impairing meat palatability. In recent years, extensive efforts have been made to enhance intramuscular fat only to yield limited success. The majority of available studies on intramuscular fat focus on the conversion of preadipocytes to adipocytes, adipocyte lipid metabolism, and hypertrophy through nutritional management. We studied the effect of lowering the extracellular calcium and retinoic acid concentration on adipocyte differentiation. Preadipocytes exposed to continuous lower calcium (lower than 3 mmol/l) and lower retinoic acid (lower than 2  $\mu$ mol/l) accumulated more cytoplasmic lipid compared with the other treatments. Differentiation was monitored by Oil Red O staining of cytoplasmic lipid and triglyceride assay. RT-PCR and Western blot analysis showed that low levels of calcium and retinoic acid upregulated the expressions of adipogenic markers PPAR $\gamma$ , C/EBP $\alpha$ , aP2, SREBP-1c and RXR. However, calreticulin, a major calcium buffering protein in the ER, was downregulated. Retinoic acid at low levels induced the expression of PPAR $\gamma$  and

reduced the expression of calreticulin. Decreased expression of calreticulin promoted adipogenesis through its effects on calcium homeostasis, causing the indirect regulation of crucial pathways affecting adipogenesis such as the calcineurin and CaMKII pathways. These results suggest that continuous exposure to low calcium and retinoic acid concentration promotes adipogenesis. Modifications on the levels of calcium and retinoic acid can be further studied to positively affect adipogenesis and to apply this knowledge in enhancing intramuscular fat accumulation in beef cattle.

## KEYWORDS

3T3-L1, calcium, retinoic acid, adipogenesis, calreticulin

## INTRODUCTION

Intramuscular fat deposition or marbling is an important trait of meat quality and has a great economic benefit for the beef industry. Intramuscular fat is known to influence tenderness, juiciness, and sensory quality of meat which makes it crucial for meat palatability and acceptability. Adipogenesis is regulated by genetic, nutritional, and environmental factors, all of which dictate the key signaling pathways regulating adipogenesis in skeletal muscle and, thus, marbling in the resulting meat. Because of this, many studies are now focusing on a different approach to enhance intramuscular fat deposition.

One area of interest is through manipulation of some micronutrients in animal feeds to promote adipocyte differentiation in beef cattle. Some of these manipulations are

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through low Vitamin A and low calcium diets. Vitamin A is a nutrient with important effects on adipose tissue development and metabolism. Retinoic acid (RA), the active form of Vitamin A, promotes or inhibits adipogenesis of preadipocytes depending on the dose (Safanova et al., 1997). In Japan and Korea, it is already known that lowering dietary Vitamin A level during the fattening stage increases marbling in beef cattle. Calcium is a versatile signaling molecule involved in the regulation of different pathways including proliferation and differentiation. Other studies also show that increasing the level of intracellular calcium stimulates adipogenesis. However, the underlying mechanism affecting adipogenesis, as resulted in the interaction among vitamin A metabolite, retinoic acid, and calcium level, is still unclear.

We report here the changes in the level of adipocyte differentiation through manipulations in the concentration of retinoic acid and calcium.

## MATERIALS AND METHODS

### 3T3-L1 cell culture and stimulation

Murine 3T3-L1 preadipocytes (Korean Cell Bank) were cultured to confluence in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% Bovine Calf Serum (BCS, Gibco) and 1% Penicillin/Streptomycin (Gibco) in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. On day 2 of post-confluence (designated as day 0), cells were induced to differentiate with DMEM supplemented with 10% Fetal Bovine Serum (FBS, Gibco), 1 μM dexamethasone (DEX, Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (IBMX, Sigma-Aldrich), and 10 μg/mL insulin (INS, Sigma-Aldrich). After 48 h (day 2), the media were replaced with DMEM supplemented with 10% FBS. The cells were subsequently re-fed every 48 h with DMEM supplemented with 10% FBS until day 8.

### Treatment of calcium and retinoic acid on 3T3-L1 cells

To investigate the effects of different concentration of calcium and retinoic acid on adipogenesis, differentiating 3T3-L1 cells were treated every two days with different concentration combination of calcium (1.8 to 10 mM) and retinoic acid (0 to 8 μM) for eight days.

### Cell viability assay

To determine the effect of different concentration combination of calcium and retinoic acid on 3T3-L1 preadipocytes, a proliferation assay was performed using the CCK-8 assay (Dojindo). Cells were seeded at a density of 1 × 10<sup>4</sup> cells/well in a 96-well plate and treated with different calcium and retinoic acid concentrations. Cells were incubated for 24 and 48 hours. CCK-8 reagent was added to the cell suspension and optical density was measured at 450nm using a micro plate reader. For the treated cells, viability is expressed as the percentage of control cells.

### Oil Red O staining

The extent of differentiation reflected by the amount of lipid accumulated was determined at day 8 by Oil Red O staining. Briefly, differentiated 3T3-L1 cells were fixed in 10% formaldehyde in PBS for 1 hour, washed with distilled water, and dried completely. Cells were stained with 0.5% Oil Red O solution in 60:40 (v/v) isopropanol:TW for 15 minutes at room temperature, washed four times with TW and dried. Differentiation was monitored under a microscope and

quantified by elution with isopropanol and optical density measurements at 490 nm.

### RNA extraction and RT-PCR

Confluent cultures of 3T3-L1 cells in 6-well plates were induced for adipocyte differentiation as previously described for 8 days. Total RNA was extracted from 3T3-L1 pellets using RNAiso Plus (Takara Shuzo Co.) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA in a 20 μl reaction using a Maxime RT PreMix Kit (iNtRON Biotechnology). PCR reactions consisted of an initial denaturing cycle at 95°C for 5 minutes, followed by 30 amplification cycles: 40 seconds at 95°C, annealing for 40 seconds (temperature ranging from 56-62°C) and extension at 72°C for 1 minute. The following oligonucleotide primers were used in RT-PCR:

Gene	Forward primer	Reverse primer
PPAR $\gamma$	5'- GAT GGA AGA CCA CTC GCA TT-3'	5'-AAC CAT TGG GTC AGC TCT TG-3'
C/EBP $\alpha$	5'-TGG ACA AGA ACA GCA ACG AG-3'	5'-TCA CTG GTC AAC TCC AGC AC-3'
aP2	5'- CCG ATC CAC TCC TTA CCT CA -3'	5'- GCC ACC GTG ACC TTG TAC TT -3'
SREBP-1c	5'- GCT GTT GGC ATC CTG CTA TC -3'	5'- TAG CTG GAA GTG ACG GTG GT -3'
Calreticulin	5'- TAC GCA CTG TCC GCC AAA T -3'	5'- GTC CAA ACC ACT CGG AAA CAG -3'
Beta-actin	5'- CAC CCC AGC CAT GTA CGT -3'	5'- GTC CAG ACG CAG GAT GGC -3'

The "housekeeping gene" beta-actin was used to verify that equal amounts of RNA were added in the PCR reaction. All gene expression values were normalized against the beta-actin expression.

### Western blot analysis

Cells were seeded in a 6-well plate and adipocyte differentiation was induced as described above with different concentration combination of calcium and retinoic acid. At day 8, protein was extracted by adding protein extraction solution (iNtRON Biotechnology). The lysates were clarified by centrifugation at 15000rpm for 15 min at 4 C and the protein content of the supernatant was determined using a modified Bradford assay. Diluted 30 μg of the protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose transfer membranes. The membranes were blocked with 5% skimmed milk and hybridized with the following primary antibodies PPAR  $\gamma$ , C/EBP  $\alpha$ , aP2, SREBP-1c, Calreticulin and beta actin (Abcam). Specific proteins were identified by further incubation of the corresponding membranes with horseradish peroxidase-conjugated secondary antibodies followed by a treatment with enhanced chemiluminescence (AB Frontier). The target proteins were exposed and detected on radiographic film.

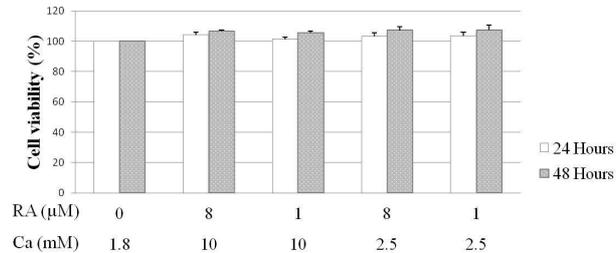
### Statistical analysis

All quantitative data are representative of at least three independent experiments and the results were expressed as means  $\pm$  S.D. Differences between means were evaluated using ANOVA test (one-way) followed by Duncan's Multiple Range Test. Differences were considered significant at p < 0.05. The statistical software package SAS v9.2 was used for the analysis.

## RESULTS AND DISCUSSION

### Effect of calcium and retinoic acid concentration on the cell viability of 3T3-L1 preadipocytes

Figure 1 shows the effect of different calcium and retinoic acid concentration combination on the cell viability of the cultured preadipocytes cells. Data show that within the experimental range of calcium and retinoic acid concentrations, the treatments did not affect the viability of cells and showed no cytotoxic effect on the cells. No significant change ( $p > 0.05$ ) on the cell viability was observed even when the concentration of calcium was increased to 10mM and retinoic acid was at  $8\mu\text{M}$  in comparison with the control. Upon microscopic analysis, no change in cell morphology was observed.

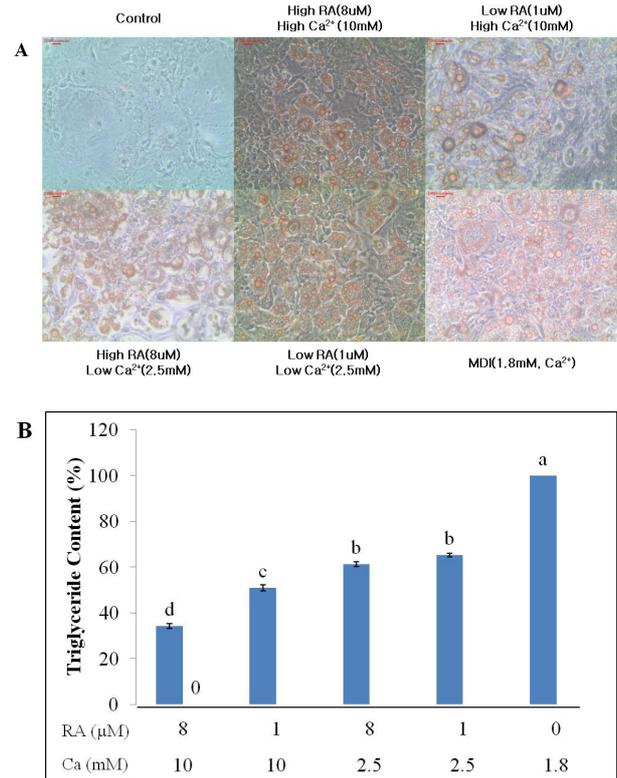


**Figure 1. Effects of calcium and retinoic acid concentration on the viability of 3T3-L1 cells incubated for 24 and 48 hours using the CCK-8 assay.** Data are means  $\pm$  SE of three replicate experiments.

#### Effect of calcium and retinoic acid concentration on adipocyte differentiation

To observe the effect of different calcium and retinoic acid concentration combination on adipogenesis in 3T3-L1 preadipocytes, post-confluent cells were induced to differentiate by exposure to MDI differentiation medium (containing IBMX, dexamethasone, and insulin) for 2 days in the presence of the corresponding treatment. Oil Red O staining of cells on day 8 showed that decreasing the concentration of calcium and retinoic acid induced increased cellular lipid accumulation in 3T3-L1 cells (Figure 2A). Cells that are exposed to continuous lower calcium (lower than 3mmol/l) and lower retinoic acid (lower than 2umol/l) accumulated more cytoplasmic lipid compared with the other treatments containing higher calcium and retinoic acid. Elution of Oil Red O stain with isopropanol (Figure 2B) showed that as the concentration of calcium and retinoic acid decreases, the triglyceride content of the differentiated adipocytes significantly increased. Our results indicate that higher extracellular calcium and retinoic concentration can inhibit the ability of 3T3-L1 preadipocytes to accumulate triglycerides and also suggests that 3T3-L1 preadipocytes can sense and respond to changes in their extracellular environment. This is in agreement with the study of Jensen et al. (2004) where an increase in the cellular calcium level induced by incubation in high calcium media inhibited the differentiation of 3T3-L1 cells. Schwarz et al. (1997) also reported that retinoic acid blocks

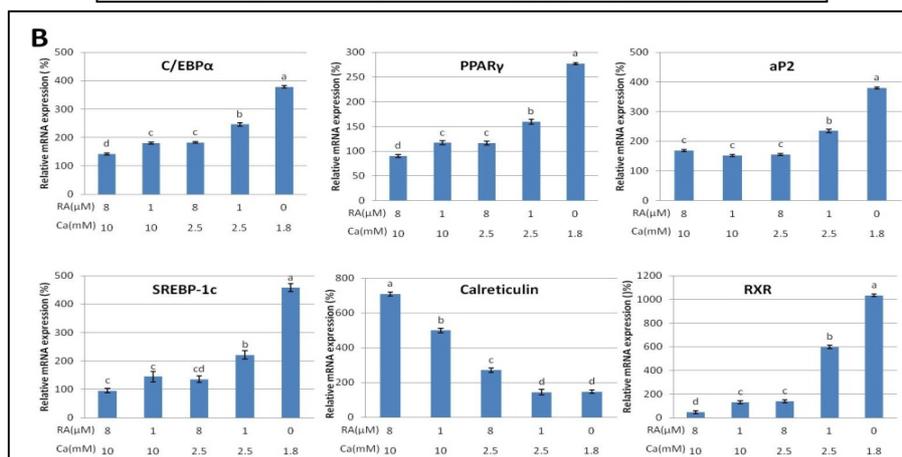
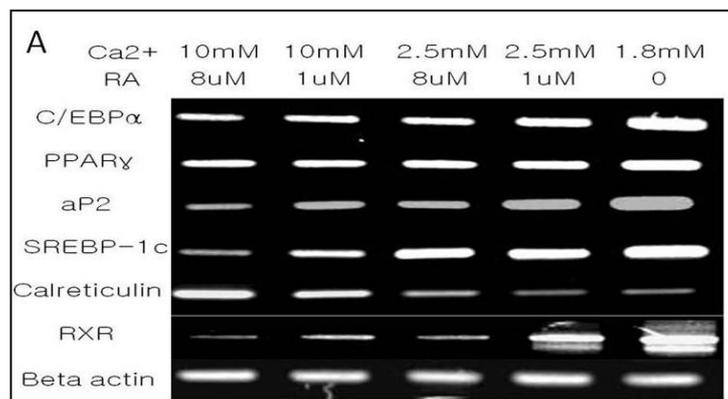
adipogenesis by inhibiting the C/EBP $\alpha$  mediated adipocyte differentiation and induction of PPAR $\gamma$ .



**Figure 2. Decreasing calcium and retinoic acid concentration promotes adipocyte differentiation in 3T3-L1 preadipocytes.** (A) Representative microscopic morphological images of adipocytes stained with Oil-Red-O after 8 days of differentiation. Cytoplasmic lipids are stained red. (B) The Oil Red O was eluted with isopropanol and quantified at 520 nm. Values are expressed as means  $\pm$  SE of three independent experiments. Means with different superscript are significantly different at  $p < 0.05$ .

#### Gene expressions associated with adipocyte differentiation

To investigate the molecular mechanism underlying the adipogenic effect of decreasing the concentration of calcium and retinoic acid, we analyzed the expression of various transcription factors associated with adipocyte differentiation. Eight days after the induction of differentiation and exposure to different concentrations of calcium and retinoic acid, the levels of mRNA expression of genes involved in adipogenesis were measured. Adipogenic differentiation was initiated by a hormonal cocktail (MDI) treatment, which accompanies the induction of various transcription factors. Figure 3 and 4 show that decreasing the level of calcium and retinoic acid from the time of adipogenic differentiation markedly increased the expression of adipogenic markers: C/EBP $\alpha$ , PPAR $\gamma$ , aP2 and SREBP-1c.



**Figure 3. Effect of calcium and retinoic acid concentration on the mRNA expressions of genes\* associated with adipocyte differentiation.** (A) Representative mRNA bands. (B) Relative mRNA expression (%). Values are expressed as means  $\pm$  SE of three independent experiments. Means with different superscript are significantly different at  $p < 0.05$ .

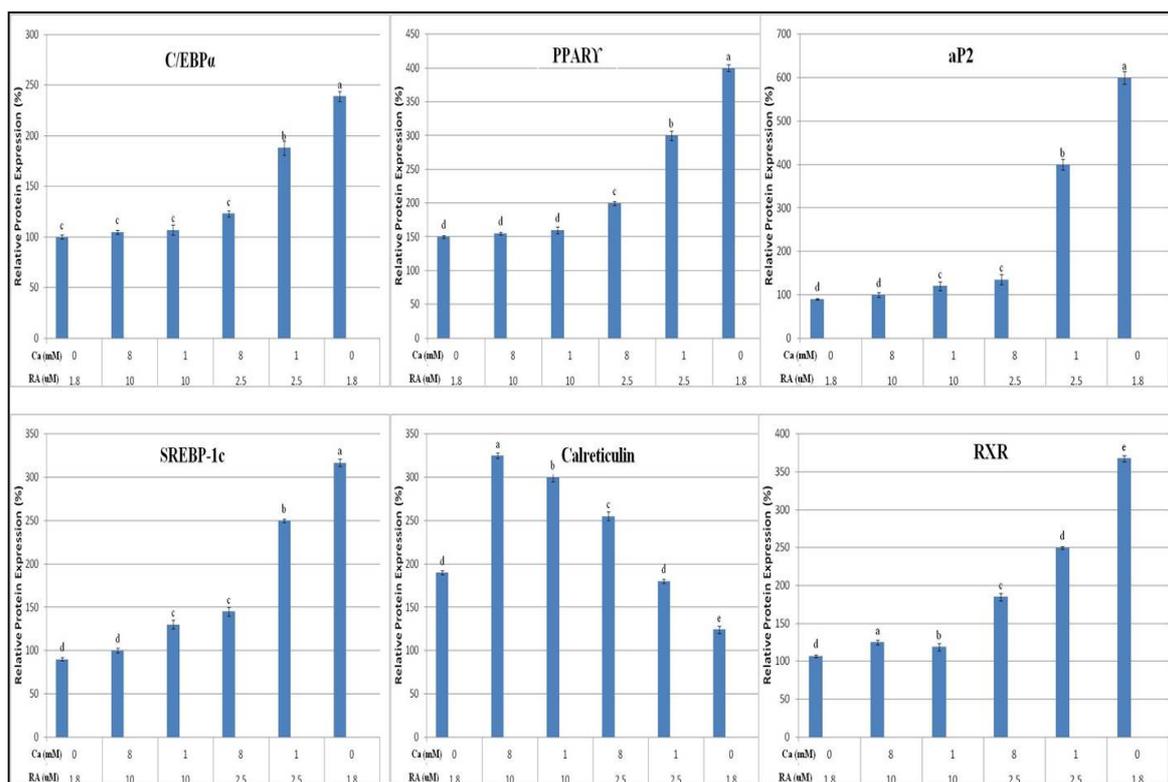
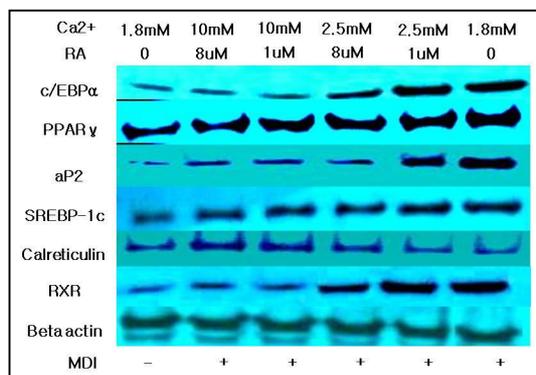
\*legends for the acronym of genes used  
 C/EBP: CCAAT/enhancer binding proteins  
 PPAR: Peroxisome proliferator-activated receptors  
 AP2: Fatty acid binding protein  
 SREBP: Sterol regulatory element binding proteins  
 CRABP-II: Cellular retinoic acid binding protein II  
 RXR: Retinoid X receptor

Adipogenic differentiation is accompanied by complex changes in the pattern of gene expression. Among the genes that undergo changes in expression during adipocyte differentiation are a cascade of transcription factors belonging to the families of CCAAT/enhancer-binding protein (C/EBP) and peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) (Gregoire et al., 1998; Wu et al., 1999; Farmer, 2006). Today, C/EBP $\alpha$  and PPAR $\gamma$  are considered the two primary transcription factors that mediate adipogenesis. PPAR $\gamma$  and C/EBP $\alpha$  have been shown to activate adipocyte-specific genes and are involved in the growth arrest that is required for adipocyte differentiation (White and Stephens, 2010). The complex process of adipogenesis starts by the production of PPAR $\gamma$ , which is controlled and activated by C/EBP $\alpha$  and SREBP-1c. C/EBP $\alpha$ , which is expressed rather late in the adipogenesis process, appears to promote differentiation in cooperation with PPAR $\gamma$  by cross regulation (Jeon et al., 2004). SREBP-1 regulates lipogenic gene expression associated with fatty acid synthesis, which leads to increased synthesis of triglycerides, and can contribute to the expression of PPAR $\gamma$  ligands (Kim and Spiegelman, 1996). AP2 also known as fatty acid binding protein 4 (FABP4) is a protein that is

expressed in adipocytes and has profound effects on insulin sensitivity and glucose metabolism and play an important role in adipocyte differentiation, is activated by PPAR $\gamma$ , C/EBP $\alpha$  and SREBP-1c (Huang et al., 2011). Following these events, the committed cells undergo terminal differentiation manifested by production of lipid droplets, as well as expression of multiple metabolic programs characteristic of mature fat cells.

These transcription factors and adipogenic markers all have important roles in the cascade of events during adipocyte differentiation. Our data (Figure 3 and 4) clearly show that decreasing the extracellular calcium and retinoic acid level enhanced the mRNA and protein expression of these important adipogenic markers and resulted in an increased degree of differentiation of the preadipocytes to mature adipose cells.

To further gain insight about the adipogenic effect of decreasing the calcium and retinoic acid concentration, we also analyzed the expression level of calreticulin. Calreticulin is an endoplasmic reticulum resident protein that affects many critical cellular functions including calcium homeostasis and inhibition of commitment to adipocyte differentiation (Szabo et al., 2008).



**Figure 4. Effect of calcium and retinoic acid concentration on the protein expressions of genes associated with adipocyte differentiation with representative bands and relative protein expression (%).** Values are expressed as means  $\pm$  SE of three independent experiments. Means with different superscript are significantly different at  $p < 0.05$ .

Meldolesi (2008) showed that PPAR $\gamma$  was found to be down-regulated in cells overexpressing calreticulin and as a consequence prevents their commitment to adipocyte differentiation. Colella et al. (2008) also proposed that calcineurin, a well-known calcium dependent phosphatase appears to mediate the inhibitory effect of calreticulin and that phosphorylation of the calcium dependent enzyme, Ca<sup>2+</sup>/calmodulin dependent protein kinase II (CaMKII) activates PPAR $\gamma$  and C/EBP $\alpha$ . Both are affected by the level of expression of calreticulin.

In association with adipocyte differentiation, many transcription factors including retinoid X receptor  $\alpha$  (RXR $\alpha$ ), C/EBP $\alpha$ , and PPAR $\gamma$  are sequentially activated to regulate the adipogenic gene expression cooperatively. RXR $\alpha$  and PPAR $\gamma$  make a complex and activate the adipogenic genes at transcriptional level by binding the promoter region directly (Schoonjans et al., 1996). With regard to RXR $\alpha$ , although some studies have been conducted in porcine adipocytes, the roles of RXR $\alpha$  in adipocyte differentiation seem to be still unclear. Retinoid X receptor (RXR) is an indispensable member of the nuclear receptor (NR)

superfamily and is considered as a key regulator in differentiation and cellular growth.

Data showed that decreasing the levels of calcium and retinoic acid significantly induced the expression of RXR and promotes adipogenesis. This is in contradiction with Ding et al. (1999) who have reported that the gene expression of RXR $\alpha$  is abundant in adipocytes but the expression level is not linked to the adipocyte differentiation but agrees with Lin et al. (2007) who suggest that RXR $\alpha$  gene plays a pivotal role in adipocyte differentiation.

Our PCR and Western blot (Figure 3 and 4) data clearly show that decreasing the extracellular calcium and retinoic acid level decrease the expression of calreticulin, thus decreasing the inhibitory effect of calreticulin on PPAR $\gamma$ . This likewise causes an indirect regulation of crucial pathways affecting adipogenesis, such as the calcineurin and CaMKII pathways, and promote adipocyte differentiation in 3T3-L1 cells.

## IMPLICATIONS

Our results showed that decreasing the concentration of extracellular calcium and retinoic acid significantly promoted adipogenic differentiation in 3T3-L1 cells with the upregulation of important adipogenic genes. Another important finding is the suppression of calreticulin expression, which is an inhibitor of adipocyte differentiation. Further studies must be conducted to fully comprehend the underlying mechanism and to apply said knowledge towards enhancing intramuscular fat deposition in beef cattle.

#### ACKNOWLEDGEMENT

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#### CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

#### CONTRIBUTION OF AUTHORS

JDC and SGH conceived and designed the experiments. JDC and SGC performed the cell experiment and analyzed the data. JDC wrote the paper. YKO and SGH revised the manuscript. SGH and YKO supervised the study. All authors read and approved the final version of the manuscript to be published.

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