Title

Effects of Biohydrogenation Intermediates on Adipogenesis and Lipogenesis of Primary Bovine Preadipocytes and Dedifferentiated Mature Adipocytes

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Effects of Biohydrogenation Intermediates on Adipogenesis and Lipogenesis of Primary Bovine Preadipocytes and Dedifferentiated Mature Adipocytes

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Abstract

Effects of Biohydrogenation Intermediates on Adipogenesis and Lipogenesis of Primary Bovine Preadipocytes and Dedifferentiated Mature Adipocytes

High-grain diets are commonly fed to increase the deposition of marbling fat in finishing beef cattle. While feeding grain-based diets is essential for producing high-quality beef, it can shift ruminal biohydrogenation pathways towards t10-18:1 and t10, c12-CLA as intermediates instead of t11-18:1 and c9, t11-CLA. The t10, c12-CLA has been consistently shown to have anti-lipogenic properties in bovine adipocyte cultures. However, bioactivities of other biohydrogenation intermediates (BHI) including t10-18:1, t11-18:1, and c9, t11-CLA are still unclear. Therefore, we sought to provide a clearer view of adipogenic and lipogenic effects of key BHI (t10, c12-CLA, c9, t11-CLA, t10-18:1, and t11-18:1) on stromovascular fraction (SVF) and dedifferentiated fat (DFAT) cells obtained from beef intramuscular adipose tissue. Confluent SVF and DFAT cells were exposed to media containing adipogenic inducers (dexamethasone, 3-isobutyl-1-methylxanthine) and 0, 5, 10, or 20 μM of BHI treatments for 6 days followed by Oil Red O staining, fatty acid, qPCR, and GPDH activity assays. DFAT-derived mature adipocytes, regardless of the BHI treatment, showed a higher supplemented fatty acid incorporation and CCAAT/Enhancer-binding protein α (C/EBP α) expression compared to SVF adipocytes. Oil Red O staining showed that t10-18:1 treatment reduced lipid accumulation compared to other BHI treatments in both SVF and DFAT adipocytes, without impacting lipogenic genes. Conversely, t10, c12-CLA significantly reduced SCD1 expression compared to other BHI treatments in both cell models, which was accompanied by reduced 18:1/18:0 and 16:1/16:0 ratios. In conclusion, our findings suggest that both t10-18:1 and t10, c12 CLA may negatively impact marbling development in beef cattle, while t11-18:1 and c9, t11-CLA are neutral in this regard. More in vitro and in vivo studies are needed to elucidate the mechanisms by which t10-

18:1 and t10, c12-18:2 may affect marbling development in beef cattle, including their potential synergistic effects on adipogenesis and lipogenesis in bovine adipocytes.

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LIST OF ABBREVIATIONS

3T3	3-day transfer, inoculum 3×105 cells
3T3-L1	3-day transfer, inoculum 3×105 cells - Swiss albino cell line of 1962
ACC	acetyl-coA carboxylase
ACP	acyl carrier protein
BHI	biohydrogenation intermediate
BSA	bovine serum albumin
C/EBP α	CCAAT/enhancer binding protein alpha
c9, t11-CLA	cis9, trans11 - conjugated linoleic acid
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CLA	conjugated linoleic acid
CO2	carbon dioxide
CoA	coenzyme A
Dex	dexamethasone
DFAT	dedifferentiated fat
DMEM	dulbecco's modified eagle medium
EGF	epidermal growth factor
EIF 3K	eukaryotic translation initiation factor 3 subunit K
ERK/MAPK	extracellular signal-regulated kinase/mitogen-activated protein kinase
FABP4	fatty acid binding protein 4
FACS	fluoresence activated cell sorting
FAME	fatty acid methyl-ether
FAP	fibro-adipogenic progenitor
FBS	fetal bovine serum
FFA	free fatty acid
G3PDH	glycerol-3-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC-FID	gas chromatography with flame ionozation detection
GLUT4	glucose transport channel 4
GPDH	glycerol-phosphate dehydrogenase
HDL	high density lipoprotein
IBMX	3-isobutyl-1-methylxanthine
KOH	potassium hydroxide
LDL	low density lipoprotein
LPL	lipoprotein lipase
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid
NADH	nicotinamide adenine dinucleotide + hydrogen
PCR	polymerase chain reaction

PDGF	platelet derived growth factor
PDGFR α	platelet derived growth factor receptor alpha
PEPCK	phosphoenolpyruvate carboxykinase
Pref-1	preadipocyte factor 12
PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
RT-qPCR	real time quantitative polymerase chain reaction
RXR	retinoid x receptor
SCD 1	stearoyl-coA desaturase 1
SFA	saturated fattu acid
SMAD	mothers against decapentaplegic
Sox9	SRY-Box Transcription Factor 9
SREBP	sterol regulatory element binding proteins
SVF	stromal vascular fraction
t10, c12-CLA	trans10, cis12 - conjugated linoleic acid
t10-18:1	trans10-18:1
t11-18:1	trans11-18:1
TGF β	transforming growth factor beta
TNF α	tumor necrosis factor alpha
TRZ	thiazolidinediones
UFA	unsaturated fatty acid
UXT	ubiquitouslty expressed prefoldin like chaperone
VDR	vitamin d receptor
WST	water-soluble tetrazolium
Zfp 423	zinc-finger protein 423

Chapter I

Comprehensive Review of Literature

Marbling fat and its importance

Marbling fat refers to the intramuscular adipose tissue that accumulates in the perimysium, which locates in between muscle fibers (Harper and Pethick, 2004). Marbling fat is important for the quality of beef because it is a major determinant of the overall flavor profile such as tenderness, juiciness, and taste. Highly marbled beef receives higher grades in the global market (e.g., Prime grade for beef from the United States, 1++ grade for Korean Hanwoo, and A5 grade for Japanese Wagyu), despite the consumer preference for high marbling varies among countries (Smith and Johnson, 2007). High consumer ratings for better-marbled beef in terms of tenderness, juiciness, and overall liking reflect the importance of marbling fat in the grading system as well (Fortin et al., 2005; Savell and Cross, 1988; Wood et al., 2004). Beside the palatability, the marbling fat offers healthfulness with high content of oleic acid, which is considered a "heart healthy" fatty acid due to its anti-cardiovascular disease properties (Kris-Etherton et al., 1999).

Marbling Fat and Beef Palatability

The palatability of beef is a combination of volatile aroma compounds and non-volatile taste compounds (peptides and free amino acid), meat juice, and dissolved fat fused in the matrix of muscle fiber, connective tissue, adipose tissue (Frank et al., 2016). Palatability has a strong positive correlation with the intramuscular (marbling) fat accumulated in the perimysium between muscle fibers. Savell and Cross (1988) reported that increased intramuscular fat in the loin muscle enhances palatability ratings, with the optimum total fat percentage ranging from 3

to 7.5 %. Similarly, a categorical comparison between Top Choice (~6.85% marbling fat) and Select (~2.95% marbling fat) grades showed that consumers experienced better tenderness with Top Choice beef regardless of muscle type (Hunt et al., 2014).

The amount of marbling fat improves the physical attributes of beef palatability. Melted marbling fat in the cooked meat juice coats the oral film to remain on the oral membrane longer and therefore allows longer flavor perception (Frank et al., 2011). Marbling fat coats connective tissues in the meat and thereby keeps meat juice in beef when cooked (Aberle, 2001), and studies consistently reported elevated meat juiciness indicators with increasing content of marbling fat (Frank et al., 2016; Hunt et al., 2014). Marbling fat enhances meat tenderness by disintegrating structure and reducing the amount of extracellular matrix in intramuscular connective tissue including perimysium and endomysuym (Li et al, 2006; Nishimura, 2009) . Marbling fat also decreases the volume of bulked muscle fiber which gives tough texture to meat (Seideman et al., 1987; Wheeler et al., 1994). This is partially indicated by the negative relationship between marbling fat content represented by USDA beef quality grade and the total moisture content of uncooked cuts because muscle fibers contain more moisture than adipose tissue (Savell et al., 1986).

Fatty acid composition

The content of marbling fat impacts beef palatability not only by affecting juiciness and tenderness but also by changing the fatty acid composition. As marbling fat accumulates in the muscle tissue, the proportion of cis-monounsaturated fatty acid (c-MUFA) increases at the expense of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA). It is consistently reported that intramuscular adipose tissue from the loin muscle of fatty carcass contained higher c-MUFA and lower SFA and PUFA than that from the leaner carcass (Indurain et al., 2006;

Sturdivant et al., 1992; Westerling and Hedrick, 1979). Likewise, the content of c-MUFA (especially oleic acid) is reported to have a strong positive correlation with palatability traits such as juiciness and tenderness, while the content of PUFA (C18:2, C18:3, and C20:4) and saturated fat (SFA; C18:0) has been associated with lower tenderness and juiciness ratings (Garmyn et al., 2011; Melton et al., 1982; Westerling and Hedrick, 1979). Overall, marbling fat positively correlates to the overall palatability of a beef cut by affecting juiciness, texture, and fatty acid profile.

Effects of Fatty Acid Composition on Beef Healthfulness

Consumption of red meats including pork and beef has been perceived to increase the risk of cardiovascular (CVD) disease for decades. Medical reports have cited the overconsumption of saturated fat as a main cause of decreased HDL: LDL ratio and atherosclerosis and blamed red meat fat, as it is one of the most saturated-fat-rich sources in human diets (Hulshof et al., 1999; Keys 1953). However, the correlation between saturated fat in beef and cardiovascular diseases is weak because stearic acid, one of the most common saturated fatty acids in beef (~34% of total saturated fatty acid) is known to have neutral effect on blood total and LDL cholesterol level (Kris-Etherton and Yu, 1997). Furthermore, myristic acid, the most potent CVD factor, presents in a low percentage (~2%) in beef fat (Daley et al., 2010). On the other hand, adverse health effects of saturated fat in marbling fat may be reverted by its high content of oleic acid. Recent studies suggest that the consumption of high-oleic ground beef increased blood levels of high-density lipoprotein (HDL) which is known to decrease the CVD risk (Adams et al., 2010; Gilmore et al., 2013; Kris-Etherton et al., 1999; Smith et al., 2020). Furthermore, bioactive fatty acids from meat fat such as trans vaccenic acid and c9,t11-conjugated linoleic acid (c9, t11-

CLA) have the potential to prevent obesity-related diseases such as type II diabetes and inflammation (Kadegowda et al., 2013; Smith et al., 2006).

Marbling Development - Adipocyte Precursors

Adipose tissue is both an energy-storing and endocrine organ that is composed of adipocytes, connective tissue, nerve tissue, stromovascular cells, and immune cells (Tang et al., 2022). Adipocytes are attached to the connective tissue matrix of adipose tissue and store fat in the form of lipid droplets in the cytosol to preserve extraneous energy (Kershaw and Flier, 2004). The development of adipose tissue is driven both by hyperplasia (cell division) of adipogenic precursors and hypertrophy (cell size growth) of mature adipocytes. In the early stage of development from the fetus to 14 months of age, adipose tissue grows mainly via hyperplasia. After 14 months of age, the main mechanism of adipose tissue growth gradually switches from hyperplasia to hypertrophy, while intermuscular fat development still depends on hyperplasia of adipogenic precursors residing in perimysial connective tissue (Hood and Allen., 1973). Due to hypertrophy being a dominant method of intramuscular adipose tissue development only in the feedlot period, providing extra energy through high-grain diets in is essential to promote marbling fat content in beef.

Adipogenic precursors of intramuscular fat are part of stromovascular cells (Hollenberg and Vost, 1969). Stromal vascular cells are a population of mural cells lying under adipose tissue vasculature including adipogenic precursors, nerve cells, endothelial cells, immune cells, and pericytes. The two main categories of adipogenic precursors used by studies are preadipocytes and fibro adipogenic precursors (FAP). Preadipocytes are defined as spindle-shaped mesenchymal stem cells that are limited to adipocyte differentiation, while FAP still possesses its multipotency to become fibroblast or preadipocytes (Cawthorn et al., 2012; Uezmi et al., 2011).

These cell populations can be separated by surface receptors with fluorescence-activated cell sorting (FACS). Uezumi et al. (2011) identified FAP from mice muscle tissue as platelet-derived growth factor receptor alpha (PDGFRα; also known as CD140a) + cells and they distinguish in adipogenic and fibrogenic capacities from muscle satellite cells. Recently, Dohmen et al. (2022) reported the combination of surface receptors (CD31-, CD45-, CD29+, CD56+) for FAP isolation by FACS from muscle satellite cells (CD31-, CD45-, CD29+, CD56-) in perimysial tissue stromal vascular fraction. The assorted FAP expressed significantly higher PDGFRα than satellite cells. However, since CD56 is a myogenic marker, the FAP cell population isolated by this group may contain preadipocytes (PDGFRα-) as well (Arrighi et al, 2015). If this cell population is additionally sorted by CD140+/-, it might be able to isolate FAP from preadipocytes as well.

Besides FAP and preadipocytes, there is another adipogenic precursor population called dedifferentiated mature adipocytes. When adipose tissue is digested by collagenase and the tissue lysate is centrifuged, mature adipocytes gather on the top layer. If these mature adipocytes are collected and cultured on the ceiling of the culture flask utilizing its buoyancy, they gradually lose lipid droplets in the cytosol and regain fibroblast-like morphology and proliferative capacity (Wei et al., 2012). Studies report that both murine and bovine-dedifferentiated mature adipocytes can differentiate into several cell types such as adipocytes, fibroblasts, chondrocytes, and myocytes (Matsumoto et al., 2008; Wei et al., 2012). It has been shown that dedifferentiation also occurs in-vivo as a self-repair mechanism (Liao et al., 2015). Indeed, the review of Song and Kuang (2019) suggests the dedifferentiation and redifferentiation from adipocytes to myofibroblast and vice versa as evidence of the de-differentiation of mature adipocytes for

adipose tissue regeneration. Overall, de-differentiated mature adipocytes might be a member of adipogenic precursors and potential contributors to intramuscular fat hyperplasia.

Acquisition of Adipogenic Precursors - Bovine Adipocyte Culture Models

Most beef marbling biology studies utilize stromovascular fraction (SVF) extracted from fresh adipose tissue or perimysium as their adipogenic precursors (Burns et al., 2012; Chung et al., 2006; Kadegowda et al., 2013; Yanting et al., 2018). SVF can be isolated by enzymatic digestion. When fresh adipose tissue is harvested via biopsy or dissected post-slaughter, it is digested by Type I collagenase solution which will strip stromal cells off from the tissue into solution. SVF cell pellets can be obtained from this solution after filtration and centrifugation. These cell pellets can be resuspended in the cell culture media, attached to the culture dish, and subcultured to achieve a higher quantity of cells for experiments. The SVF is often subcultured when it reaches 70-90% confluence on the cell culture dish (Mehta et al., 2019).

Other than enzymatic digestion, SVF can also be obtained by primary explant culture. In primary explant culture, a fragment of fresh adipose tissue simply submerges in culture media and attaches to the surface of the cell culture dish. Attachments of tissues can be stimulated by polylysine or fibronectin treatment on the surface. At least after 4 days, proliferative cells that are attached to the surface start to outgrow the tissue. Similar to enzymatic digestion, outgrown cells can be grown until 70~90% confluence and subcultured to harvest more cells (Jing et al., 2011). This method is useful for small amounts of tissues because enzymatic and mechanical digestion can compromise cell yield and viability (Freshney, 2006).

Another cell source, which is dedifferentiated fat cells (DFAT cells), can be obtained by the ceiling culture mentioned above. Since mature adipocytes are buoyant, researchers usually

add a full load of culture media into the culture flask to let mature adipocytes float on the top and touch the ceiling to attach to the culture surface. When the dedifferentiation process is complete, flasks are turned upside down and culture media is refreshed to grow and subculture the cells. Subsequent steps are the same as enzymatic digestion and primary explant culture (Wei et al., 2013). As adipocytes are the only buoyant and adhesive cells in the culture flask, dedifferentiated fat cells exhibit higher homogeneity than stromovascular cells, despite their lower proliferative capacity (Matsumoto et al., 2008).

Comparison between DFAT cells and SVF

Although they share the same origin, DFAT cells and SVF express significant differences in terms of cell profile, adipogenic capacity, and lipogenic potential. Matsumoto et al. (2008) revealed that SVF contained smooth muscle cells, lymphocytes, monocytes, and endothelial cells, while DFAT cells had a negligible proportion of these cells. From human adipose-derived SVF and DFAT cells, Watson et al. (2014) and Fujii et al. (2022) showed that DFAT cells exhibited higher lipid accumulation measured by both Oil Red O and Alizarin Red staining. There are many published studies covering bovine DFAT cells (Wei et al., 2012; Wei et al., 2013), but published data on the direct comparison of bovine DFAT cells and SVF are very limited.

Adipogenic Differentiation Protocol

Regardless of adipogenic precursor isolation methods, in-vitro adipogenic differentiation is characterized by two steps: differentiation and maturation. First, cells grow until 100% confluence followed by an extra 24~48 hours for growth arrest. Then cells are exposed to a differentiation cocktail containing dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), and insulin. Additional components like glucose or acetate as an extra energy source, PPARγ

agonists such as thiazolidinediones (troglitazone, pioglitazone, pioglitazone, etc.), and vitamins (vitamin A, vitamin E) may be added to the differentiation cocktail to enhance adipogenic differentiation (Zhao et al., 2019). The duration of differentiation step is commonly 48 hours, but some studies extend it to 4-6 days to enhance the accumulation of lipid droplets (Yanting et al., 2018; Huang et al., 2012; Zhao et al., 2019). After differentiation, cells undergo maturation in maturation media containing basal growth media supplemented with insulin for 6-12 days to accumulate lipid droplets. Extra components like energy sources and PPARγ agonists may also be added to maturation media depending on the differentiation protocols. The lipid droplets can be stained with Oil Red O for morphological analysis and relative lipid content quantification (Cholewiak et al., 1968). Alongside Oil Red O, hematoxylin can be used to stain cell nuclei for a more detailed image (Grant et al., 2008).

Process of Adipogenesis and Lipogenesis

Adipogenesis is the process by that spindle-shaped multipotent stem cells restrict their fate to adipogenic lineage, transform into rounder cell morphology, and accumulate lipid droplets in cytosol using glucose or acetate as precursors to become a mature adipocyte (Gregoire et al., 1998). The term adipogenesis often includes sequential activation of transcription factors for cell fate restriction and lipogenesis which usually only refers to the synthesis of lipid droplets in the cytosol (Ali et al., 2013).

At the beginning of adipogenesis, proliferative adipogenic precursors withdraw from the cell cycle and stop mitotic division (Lefterova and Lazar, 2009). This growth arrest accompanies by the upregulation of adipogenic genes including PPAR γ and C/EBP α (Altiok et al., 1997; Umek et al., 1991), and repression of preadipocyte markers that are known to inhibit adipogenesis such as preadipocyte factor-1 (Pref-1) and PDGFR α (Petruschke et al., 1994).

These changes in gene expression further stimulate the expression and activation of adipogenic genes such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), fatty acid binding protein 4 (FABP4), and stearoyl-CoA desaturase 1 (SCD 1) for subsequent maturation and lipid accumulation.

Adipogenic Genes

Preadipocyte markers.

The initial signal of adipogenesis is characterized by the repressing expression of the preadipocyte marker pref-1. Pref-1 is an epidermal growth factor (EGF) repeat-containing transmembrane protein that can be activated in a soluble form with tumor necrosis factor α (TNF α). Activated pref-1 stimulates extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway and then upregulates Sox9 expression to keep adipogenic precursors undifferentiated(Smas and Sul, 1993). The inhibitory effect of pref-1 on adipogenesis is repeatedly shown by either overexpression or exogenous treatment of soluble pref-1 (Smas et al., 1997; Smas and Sul, 1993). When 3T3-L1 preadipocytes are exposed to differentiation media containing dexamethasone, pref-1 expression decreases to a negligible level and signals the beginning of adipogenesis (Sams et al., 1999).

As discussed earlier, PDGFR α is another surface receptor marker that defines fibro-adipogenic precursors (FAP), one of the adipogenic precursors that are highly enriched between skeletal muscles (Uezmi et al., 2011). However, the review of Cawthorn et al. (2012) suggested that PDGFR α is a marker of adipose-derived stem cells that contains more potential cell fate than fibroblast and adipocytes. Regardless of what cells PDGFR defines, it is reported that PDGFR expression decreases after exposure to an adipogenic induction medium in 3T3-L1 studies (Vaziri and Faller, 1996; Summers et al., 1999). As platelet-derived growth factor

(PDGF) inhibits adipogenesis by suppressing PPAR γ expression, repression of PDGFR may be necessary (Artemenko et al., 2005; Camp et al., 1997). Compared to the 3T3-L1 cell line, the gene expression analysis of Mizoguchi et al. (2010) suggested that PDGFR α is upregulated after clonal bovine intramuscular preadipocyte from Japanese Black cattle is differentiated into mature adipocytes with induction medium. This result is consistent with the finding of Martins et al. (2015) which demonstrates higher PDGFR α expression by western blot from a highly marbled breed (finishing Black Angus) than a low-marbled breed (Nellore). Regardless of whether this high expression of PDGFR α is from higher content of preadipocytes or mature adipocytes, it is clear that PDGFR α has a positive correlation to the adipogenic potential of precursor cells.

Different from pref-1 which works as a gatekeeper of adipogenesis, Zfp 423 is enriched in highly adipogenic precursors. Ectopic expression of Zfp 423 in non-adipogenic 3T3 cell lines allows adipogenic differentiation by stimulating expression of PPAR γ. Conversely, reduction of Zfp 423 in 3T3-L1 preadipocytes decreases differentiation capacity. Although the full mechanism of Zfp 423 in adipogenesis is not elucidated, it is suggested that Zfp 423 activates SMAD proteins to sensitize the BMP signaling cascade and stimulate PPAR expression (Gupta et al., 2010). Huang et al. (2012) reported that primary bovine preadipocytes with high adipogenic potential show higher expression of Zfp 423 than low adipogenic preadipocytes, indicating the regulatory role of Zfp 423 is not limited by species. Unlike pref-1, the expression of Zfp 423 does not dramatically decrease after differentiation, suggesting that Zfp 423 indicates the level of adipogenic potential but not the timing of adipogenesis (Gupta et al., 2010; Huang et al., 2012).

Another gene that indicates the level of adipogenic potential of preadipocytes is TGF-β. TGF-β is a regulator gene that may cause fibrosis if the expression is uncontrolled (Yu and

Stamenkovic, 2000). TGF-β is reported to be a potent inhibitor of adipogenesis in many studies (Petruschke et al., 1994; Serrero et al., 1991). The suggested mechanism of TGF β inhibiting adipogenesis leads adipogenic precursors to fibroblast lineage instead of adipogenic lineage by activating the SMAD pathway and stimulating extracellular matrix secretion such as collagen and fibronectin (Kennedy et al., 2008). The study of Huang et al. (2010) showed that TGF β expression is higher in low adipogenic bovine preadipocytes than in high adipogenic ones.

Adipogenic & lipogenic genes – major transcription factors.

The most apparent signal of adipogenesis is the upregulation of PPAR γ and C/EBP α. Peroxisome proliferator-activated receptor (PPAR) is a type of nuclear receptor protein that regulates a variety of gene expression in different tissues such as adipose tissue, liver, and muscle, and PPAR γ is a PPAR subtype that is specific to adipose tissue (Houseknecht et al., 2002). Different from PPAR γ, C/EBP α, another transcription factor, is not adipose tissue-specific, but its expression is known to upregulate after PPAR γ and before other adipogenic and lipogenic genes (Gregoire et al., 1998). The report of Rosen et al. (2002) suggests that while PPAR γ is essential for adipogenesis, C/EBP α alone without PPAR γ expression is not sufficient to lead to adipogenesis. When expressed, PPAR γ and C/EBP α induce expression and activation of one another to regulate subsequent adipogenic genes such as fatty acid binding protein 4 (FABP4), lipoprotein lipase (LPL), stearoyl CoA desaturase 1 (SCD1), glucose transporter type 4 (GLUT4), phosphoenolpyruvate carboxykinase (PEPCK), and leptin (Gregoire et al., 1998). In cell culture conditions, the differentiation inducers such as IBMX and insulin are used to raise cAMP and protein kinase activities to stimulate expression of PPAR γ and C/EBP α (Kim et al., 2010), and the thiazolidinediones (TRZ) bind to and activate PPAR γ for subsequent transcriptions of adipogenic genes (Chiarelli and Di Marzio, 2008). Due to their important roles

in adipogenesis, researchers present the upregulation of PPAR γ and C/EBP α to seek evidence of adipogenesis (Choi et al., 2014; Chung et al., 2006; Huang et al., 2010; Yanting et al., 2018).

Adipogenic & lipogenic genes – lipid accumulation regulators.

After adipogenic differentiation, lipid droplets start to accumulate in the cytosol of adipocytes. Lipid accumulation is driven by two processes: de novo lipogenesis (formation of fatty acids from acetyl-CoA subunits produced from non-fat sources such as carbohydrates and protein) and direct uptake of fatty acids from plasma (Sanders et al., 2016). When PPAR γ and C/EBP α are upregulated, two important genes for de novo lipogenesis: ACC and FAS are upregulated (Paulauskis and Sul., 1988). Fatty acid synthesis begins with the formation of a butyryl-Acyl carrier protein (ACP) from acetyl-CoA and malonyl CoA. Then, FAS adds 2 carbons of malonyl-CoA and leaves a carbon dioxide to butyryl-ACP and elongates it until 16 carbons. Alongside FAS, ACC provides malonyl-CoA by adding a carboxyl group to acetyl-CoA (Wakil et al., 1983). Studies report that FAS expression is regulated by PPAR γ which is upregulated by the SREBP signaling pathway in goat and buffalo mammary epithelial cells (Xu et al., 2016; Fan et al., 2021). Furthermore, ACC1 expression and activity is stimulated by insulin release from high glucose level (Andreolas et al., 2002; Witters et al., 1988). Due to their roles in de novo lipogenesis, scientists consider that FAS and ACC are important genes for the later stage of adipogenic differentiation.

Other than fatty acid synthesis by FAS and ACC, GLUT4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) contribute to de novo lipogenesis by substrate supply. GLUT4 is an insulin-sensitive glucose transport channel that imports glucose from plasma into cells. When adipogenic precursors are differentiated, expression of GLUT4 increases alongside PPAR γ expression to import more glucose for acetyl-CoA synthesis via glycolysis and

anaerobic fermentation (van Harmelen et al., 2007; Nicholls et al., 2012). During glycolysis, GAPDH converts glyceraldehyde-3-phosphate (G3P) to 1, 3-biphosphoglycerate (1, 3 BPG), which is a rate-limiting step of acetyl-CoA synthesis (substrate of acetyl-CoA synthesis) (Shestov et al., 2014). Due to its constant expression in animal cells for cellular respiration, GAPDH is used as a housekeeping gene for western blot and quantitative PCR (Barber et al., 2005; Wu et al., 2012). On the other hand, since GAPDH is involved in acetyl-CoA production, increased GAPDH activity is a commonly used index of insulin sensitivity enhancement and adipogenic differentiation (Alexander et al., 1985; Singh et al., 2006). The enzyme works right before GAPDH, glycerol-3-phosphate dehydrogenase (G3PDH or GPDH) is also commonly used to assay adipogenic differentiation (Grant et al., 2008; Yanting et al., 2018).

The initial fatty acid product of de novo lipogenesis is palmitic acid (C16:0), but it can be further elongated by fatty acid elongase and desaturated by desaturases to become unsaturated fatty acid. The important desaturase in beef fat synthesis is SCD-1, because it is an endoplasmic reticulum-bound enzyme that introduces a double bond in the Δ9 position of saturated fatty acid to synthesize monounsaturated fatty acid such as oleic acid (c9-18:1) and palmitoleic acid (c9-16:1), which in turn improves fatpalatability and healthfulness (Ascenzi et al., 2021; Garmyn et al., 2011; Melton et al., 1982; Westerling and Hedrick., 1979). SCD-1 expression is regulated by PPAR γ (Miller et al., 1996), and inhibition of SCD-1 reduces de novo lipogenesis (Choi et al., 2014; Chung et al., 2006; Kadegowda et al., 2013), indicating that expression and activity of SCD-1 are important for both quality and quantity of marbling fat.

Lipid droplet accumulation is driven not only by de novo lipogenesis from carbohydrates but also by free fatty acid uptake from plasma. When adipogenic precursors are differentiated, expression of LPL increases alongside PPAR γ upregulation (Schoonjans et al., 1996).

Lipoprotein lipase is an extracellular enzyme that extracts triglyceride from low-density lipoprotein (LDL) in circulation into cells (Eckel et al., 1989). After triglyceride is hydrolyzed into free fatty acids, FABP4 (also known as aP2) binds to and transport them into different compartments of cells, including lipid droplet in the cytosol (Furuhashi and Hotamisligil, 2022). The regulation of FABP4 is regulated by PPAR γ (Rosen et al., 2000), and a change in FABP4 expression may directly affect adipogenesis and lipogenesis as well. Both the study of Wang et al. (2022) and Zhang et al. (2017) report that overexpression of FABP4 in human skeletal muscle and bovine longissimus muscle stromovascular cells leads to enhanced lipogenesis while inhibition of FABP4 reduced lipogenesis, indicating that FABP4 is one of the regulators of PPAR γ adipogenic signaling pathway. Garin-Shkolnik et al. (2014) reported that adipocytes from FABP4-knockout mice expressed higher PPAR γ expression, indicating that FABP4 may affect PPAR γ expression in a negative feedback loop.

Factors Affecting Adipogenesis & Lipogenesis Processes in Beef Cattle

Genetics and gender

The type of feed (i.e. forage vs. grain-based) is an important determinant of intramuscular fat deposition. However, genetic variation among cattle breeds influences intramuscular fat deposition in beef as well. The review by Park et al. (2018) stated that the marbling score of beef is highest in first Wagyu, second Hanwoo, third Angus, fourth Hereford, and fifth Brahman when they are grain-finished at feedlots. The difference in marbling content among breeds is in part related to the number of FAPs (PDGFRα+) present in bovine muscles (Martins et al., 2015). Roudbari et al. (2020) revealed that marbled breeds enrich MAPK signaling pathways (associated with cellular activities like proliferation, differentiation, survival, and death), which may contribute to active hyperplasia of intramuscular precursors. Furthermore, the breeds

belonging to Bos Taurus tend to have higher marbling scores and meat tenderness than the breeds belonging to Bos Indicus, highlighting the effects of genetics on beef quality (Martins et al., 2015; Wheeler et al., 1994). Differences in genetics cannot be easily substituted by nutrition and management, since the heritability of marbling score ranges from 0.3 to 0.57 and it is especially high in highly marbled breeds like Hanwoo (0.64) and Wagyu (0.4~0.55) (Utrera and Van Vleck, 2010). Therefore, selecting semen and sire is as important as feed rations and management for beef marbling score.

It has been shown that steers and heifers possess higher marbling scores than bulls (Marti et al., 2013; Park et al., 2002; Moreira et al., 2003; Prado et al., 2009), even though bulls often show the highest growth rate (Seidemen et al., 1982; Rotta et al., 2009). Bulls have higher hot carcass weight due to anabolic hormones released from testicles (Lee et al., 1990). Testosterone from testicles was reported to stimulate myogenesis and suppress adipogenesis of muscle-derived stem cells (Singhet et al., 2003). Compared to bulls, increased secretion of estrogen in steers is reported to stimulate GAPDH activity (Oh et al., 2005). Furthermore, progesterone released from cows may stimulate adipogenic genes such as PPAR γ and SREBP to improve fat accumulation in meat (Lacasa et al., 2001; Yang et al., 2016).

Nutrition

Marbling fat is influenced not only by excess energy intake but also by micronutrients such as vitamins and minerals. Marbling fat deposition improves when young calves receive vitamin A supplements (Harris et al., 2018). This is possibly due to vitamin A supplement increasing Zfp 423 expressing adipogenic precursors in muscle tissue (Maciel et al., 2022). However, vitamin A supplementation during the feedlot stage has been shown to inhibit adipogenesis as vitamin A competes for the retinoid-X-receptor (RXR) that is required for PPAR

γ activation (Pyatt and Berger, 2005). Oka et al. (1999) specifically recommended that vitamin A restriction during 14~23 months of age improves marbling scores without causing adverse health effects. Other than vitamin A, vitamin D with vitamin D receptor (VDR) also restricts adipogenic differentiation by competing with PPAR γ for RXR (Hida et al., 1999; Wang et al., 2016), although vitamin D restriction does not lead to significant marbling score improvement (Pickworth et al., 2012). Vitamin C is suggested to improve the marbling score of the Japanese Black Cattle and Angus breed (Ohashi et al., 2000; Pogge et al., 2013). Vitamin C supplementation is shown to depress the ERK signaling pathway and upregulate collagen IV (a marker of adipogenic differentiation) in 3T3-L1, but the mechanism in-vivo is still unknown (Aratani et al., 1988; Liu et al., 2017).

Minerals can influence marbling accumulation as they work as secondary messengers in different cell signaling pathways involved in adipogenesis (Newton et al., 2016). Tanaka et al. (2001) showed that zinc supplementation to 3T3-L1 cells increases GAPDH activities in a dose-dependent manner, but it did not affect marbling score when added to beef cattle diets. Yano et al. (2004) also stated that zinc supplementation on bovine stromovascular cells improved GAPDH activity regardless of with or without insulin. However, zinc supplementation in-vivo shows inconsistent effects on marbling scores (Malcolm-Callis et al., 2000; Spears and Kegley, 2002). Magnesium supplementation is consistently reported to improve marbling in pork (Apple et al., 2000; Swigert et al., 2004), but the effect of magnesium is inconsistent in beef (Coffey and Brazle, 1996; Ramirez and Zinn, 2000) even though serum magnesium level is proportional to a marbling score of Japanese Black steers (Adachi et al., 1999). Compared to zinc and magnesium, increased dietary sulfur has been consistently reported to decrease the yield and marbling score of feedlot cattle (Gibson et al., 1988; Pogge et al., 2013; Richter et al., 2012).

Management

Nutritional management at an early age can impact marbling deposition. Since adipose tissue grows by hyperplasia during early development, producers usually choose early weaning age to maximize space for future fat accumulation via hypertrophy (Du et al., 2010). It is consistently reported that early weaning age combined with a high-grain diet increases the marbling score (Meyer et al., 2005; Moisa et al., 2014; Walcott et al.,2010; Wertz et al., 2002). It is suggested that early weaning with high grain diet stimulates PPAR γ and C/EBP α activation to lead fibro-adipogenic precursors in muscle tissue toward adipogenic lineage (Park et al., 2018). However, the effect of early weaning is less in breeds with high marbling such as Wagyu than in other breeds such as Angus (Wertz et al., 2002). The timing of feedlot entry also impacts the marbling of beef cattle. Producers feed calves a high-grain diet right after weaning (i.e. calf-fed) or allow them to graze on pastures until 12 months of age (i.e. yearling fed) depending on feed and land availability. These methods do not show different marbling scores in the same body weight, but the MUFA: SFA ratio of beef fat is lower in yearling-fed cattle than in calf-fed cattle due to lower expression of SCD-1 (Brooks et al., 2011a and b). This may impact the quality of marbling as MUFA:SFA ratio is one of the major determinants of beef fat palatability.

Biohydrogenation

Biohydrogenation is a metabolic process in the rumen of ruminant animals that converts unsaturated fatty acids (UFA) to SFA. As a high concentration of UFA from plant materials compromises the viability of microbial species, rumen microbiomes are evolved to convert UFA to SFA through biohydrogenation process (Polan et al., 1964). Due to the biohydrogenation in the rumen, the fatty acid composition of animal products from ruminants such as cattle, sheep,

and goats have a higher saturated fat content than that from non-ruminant animals such as pigs and chickens (National Research Council, 1976).

The two major PUFA ruminant diets include linoleic acid (18:2n-6; cis-9, cis-12 18:2) and α-linolenic acid (18:3n-3; cis-9, cis-12, cis-15 18:3) (Alves et al., 2021). Biohydrogenation of these two PUFA results in production of several intermediates containing trans double bonds that can escape from the rumen and enter into circulation following postruminal absorption, and accumulate in tissues and milk (Griinari et al., 2000). Biohydrogenation intermediates (BHI) are the major source of trans fatty acids found in meat and milk from ruminant animals.

Normal vs. Trans10-shifted Biohydrogenation Pathways

When ruminants consume forage-based diets, linoleic acid in the feeds gets isomerized into c9, t11-conjugated linoleic acid (c9, t11-CLA). The resulting c9, t11-CLA is then hydrogenated to t11-18:1 and subsequently to 18:0 (stearic acid). Meanwhile, α-linolenic acid first gets isomerized into c9, t11, c15 -18:3 and then hydrogenated to t11,c15-18:2, t-11 18:1, and 18:0 consecutively. The resulting 18:0 and a portion of BHI are absorbed in the small intestine, enter the circulation,and accumulate in meat and milk. The absorbed t11-18:1 can further be desaturated back to c9, t11-CLA by Δ-9 desaturase (SCD1) in body tissues (Alves et al., 2021).

On the other hand, when ruminants consume grain based-diets such as feedlot rations, the major t18:1 isomer becomes t-10 18:1 instead of t11-18:1. In the t10-18:1 pathway, linoleic acid is isomerized to t10, c12-CLA which then hydrogenated to t10-18:1 and 18:0 consecutively. The α-linolenic acid in the "t10 shifted" pathway first get isomerized into t-10,c12,c15-18:3 and then sequentially hydrogenated to t10,c15-18:2, t-10-18:1, and C18:0. . This elevated ratio of t10-18:1 to t-11 18:1 in milk, tissues or blood is defined as a "t10 shift" which is common in high-grain

fed ruminants such as feedlot sheep and cattle (Alves et al., 2021). The t-10 shift is rarely seen in forage-fed/grazing ruminants (Umberger et al., 2002; Millen et al., 2011).

Effects of Trans10 biohydrogenation Intermediates on mammary lipogenesis and a milk fat

Unlike feedlot beef cattle, dairy cattle rarely fed grain-based feeds because the t10 shift is associated with milk fat depression. Milk fat depression is indicated by a reduction in milk fat content and percentage (commonly a decrease of 0.2% or more) without affecting milk protein composition (Bauman and Griinari, 2000). Baumgard et al (2000) and Peterson et al. (2003) have shown that milk fat depression is primarily caused by t10,c-12-CLA produced during "t10 shifted" biohydrogenation pathway. The t10,c-12-CLA has been suggested to inhibit milk fat synthesis because of its potent antilipogenic properties (Harvatine et al., 2009).

Meanwhile, it is still controversial whether other BHI such ast- t10-18:1 have anti-lipogenic properties. The principal component and multivariate analysis of 3 lactating cow feeding studies also state that t-10 18:1 shows a negative relationship to milk fat percentage, indicating the potential anti-lipogenic properties of this BHI in the mammary gland (Kadegowda et al., 2008). On the other hand, abomasal infusion of t10-18:1 did not cause milk fat depression in cows (Lock et al., 2007). Furthermore, t10, c15-18:2 (another 18:2 precursor of t10-18:1) does not exhibit anti-lipogenic activity in the 3T3-L1 adipocytes(Vahmani et al., 2016). Judging by published literature, although t10, c-12-CLA may not be the only cause of milk fat depression, it may be the most potent inhibitor of milk fat synthesis. To further elucidate the effects of other BHI on milk fat synthesis, it would be necessary to test individuals or combinations of BHI via either in-vivo abomasal infusion or in-vitro bovine mammary epithelial cell culture.

Effects of trans10, cis12-CLA on Adipogenesis & Lipogenesis Processes in Bovine Adipocytes

Effects of t10, c12-CLA on adipogenesis and lipogenesis in adipocytes have studied using primary bovine preadipocyte cultures. In 2006, Chung et al. (2006) showed that 40 μM of t10, c12-CLA decreased the expression of PPAR γ, SCD, LPL, TNF α, and Pref-1 in differentiated stromovascular cells isolated from bovine perirenal adipose tissue, even though they were differentiated with arginine supplement which is known to promote adipogenic differentiation (Yan et al., 2002). Later, Kadegowda et al. (2013) reported that 50 μM of t10, c12-CLA treatment does not change the differentiation level of adipocytes measured by GAPDH activity, but it significantly depresses the expression of SCD, ACC, and FAS, and reduces C16:0 synthesis de novo fatty acid synthesis from acetate. In this study, downregulation of PPAR γ was less pronounced than the report of Chung et al, which could be due to the difference in stromovascular cell sources (perirenal vs. intermuscular adipose tissue of longissimus muscle) used in these studies. The report of Choi et al. (2018) utilizing ex-vivo bovine adipose tissue cultures also found reduced of SCD and PPAR γ expression and de novo lipogenesis caused by CLA supplements. Besides depression of adipogenic and lipogenic genes, a couple of studies reported that t10,c12-CLA enhances expression of CPT 1β, a key enzyme involved in fatty acid β-oxidation, which could lead to reduced lipid accumulation in adipocytes (Kadegowda et al., 2013; Choi et al., 2014).

A few studies report the effects of other fatty acids on adipogenesis and lipogenesis in bovine adipocyte cultures. Chen et al (2018) reported that culturing with linoleic acid (C18:2n-6) resulted in decreased expression of ACC1 and HSL compared to saturated fatty acids (14:0, 16:0, 18:0), c-monounsatuared fatty acids (oleic acid), and the control (no fatty acid). Choi et al.

(2014) also reported that t-11-18:1 treatment increased PPAR γ while it decreased expression of SCD-1. Meanwhile, t10-18:1 has not been tested on any primary bovine cell culture study yet.

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Chapter II

Effects of Biohydrogenation Intermediates on Adipogenesis and Lipogenesis in Bovine Intramuscular Adipocytes

Abstract

Trans10, cis12 conjugated linoleic acid (t10, c12-CLA) has been consistently shown to have anti-lipogenic properties in bovine adipocyte cultures. However, bioactivities of other biohydrogenation intermediates (BHI) including t10-18:1, t11-18:1, and c9, t11-CLA is still unclear. Therefore, we sought to illustrate a clearer view of adipogenic and lipogenic effects of key BHI (t10,c12-CLA, c9, t11-CLA, t10-18:1, and t11-18:1) on stromovascular fraction (SVF) and dedifferentiated fat (DFAT) cells obtained from beef intramuscular adipose tissue. Confluent SVF and DFAT cells were exposed to media containing adipogenic inducers (dexamethasone, 3-isobutyl-1-methylxanthine) and 0, 5, 10 or 20 μM of BHI treatments for 6 days followed by Oil Red O staining, fatty acid, gene expression analysis and GPDH activity assays. DFAT-derived mature adipocytes, regardless of the BHI treatment, contained higher concentrations of supplemented fatty acids and CCAAT/Enhancer-binding protein α (C/EBP α) expression compared to SVF adipocytes. Oil Red O staining showed that t10-18:1 treatment reduced lipid accumulation compared to other BHI treatments in both SVF and DFAT adipocytes, without impacting lipogenic genes. Conversely, t10, c12 CLA significantly reduced SCD1 expression compared to other BHI treatments in both cell models, which was accompanied by reduced 18:1/18:0 and 16:1/16:0 ratios. In conclusion, our findings suggest that both t10-18:1 and t10, c12-CLA may negatively impact marbling development in beef cattle, while t11-18:1 and c9, t11-CLA are neutral in this regard. More *in vitro* and *in vivo* studies are needed to elucidate the mechanisms by which t10-18:1 and t10, c12-18:2 may affect marbling development in beef

cattle, including their potential synergistic effects on adipogenous and lipogenesis in bovine adipocytes.

Introduction

In ruminant animals such as cattle, sheep and goats, conjugated linoleic acid (CLA) and trans-18:1 (t-18:1) isomers are produced by rumen microbes during biohydrogenation of dietary polyunsaturated fatty acids (PUFAs), and these can be incorporated into tissues and milk (Lorenço et al., 2010; Dugan et al., 2011). When cattle are fed forage-based diets, t11-18:1 and cis(c)9, t11-CLA are respectively the main t-18:1 and CLA isomers produced in the rumen. However, when a grain-based diet is fed, ruminal biohydrogenation pathways shift towards producing t10 double bond containing CLA and t-18:1 isomers (t10-18:1 and t10, c12-CLA respectively). This phenomenon has been referred to as the "trans-10 shift" which is the predominant biohydrogenation pathway in ruminants fed grain-based diets including feedlot cattle (Alves et al., 2021).

Using synthetic/pure CLA isomers, it has been demonstrated that the t10, c12-CLA, but not c9, t11-CLA, exerts potent anti-lipogenic effects in cell culture (3T3-L1 adipocytes) and mouse models. Similarly, in primary bovine adipocytes, t10, c12-CLA, has been shown to strongly inhibit the delta 9 desaturation by inhibiting SCD1 expression and activity leading to significant reduction in oleic acid which is the primary product of SCD1 and the predominant fatty acid present in intramuscular (marbling) fat. Based on these findings , the authors suggested that the rumen-derived t10, c12-CLA may reduce marbling fat development in beef cattle (Choi et al., 2014; Kadegowda et al., 2013; Smith et al., 2009. However, the t10, c12-CLA concentrations (doses) used in these studies are much lower than that to which the tissues would be exposed in vivo. In contrast to t10, c12-CLA whose concertation is extremely low (<0.1% of

total fatty acids) in plasma and tissues of ruminants including feedlot cattle, t10-18:1 can reach up to 10% of total fatty acids in adipose tissues of feedlot cattle. Interestingly, several studies in dairy cows found t10-18:1 to be negatively correlated with milk fat concentration (Bauman and Griinari, 2003; Metamoros et al., 2020). (Bauman and Griinari, 2003). Thus, we sought to determine whether t10-18:1, because of the presence of t10 double bond, would exert similar anti-lipogenic properties in primary bovine adipocyte cultures.

To test the potential anti-lipogenic properties of t10-18:1, we chose two cell culture models: stromal vascular fraction (SVF) and dedifferentiated mature adipocytes (DFAT). SVF-derived preadipocytes have commonly been used as a model to study beef marbling biology for its easiness and proliferative potential (Chung et al., 2006; Kadegowda et al., 2013; Yanting et al., 2018; Yu et al., 2022). However, the stromal vascular fraction has limitations because it contains not only preadipocytes but also other cells such as fibro-adipogenic precursors (FAP), immune cells, pericytes, and endothelial cells (Hollenberg and Vost, 1969).

Dedifferentiated mature adipocytes (DFAT) are mature adipocytes that are dedifferentiated into lipid-free multipotent cells. They are known to be a more homogeneous cell population than SVF and readily redifferentiate into the adipogenic lineage (Matsumoto et al., 2008). Furthermore, DFAT from human adipose tissue has been shown to have a higher lipogenic potential than SVF (Fujii et al., 2022; Watson et al., 2014). However, we are not aware of any published study that compares the adipogenic potential of bovine SVF and DFAT cells, or their lipogenic response to bioactive fatty acids such as CLA.

The objectives are of this study are: 1) investigate the bioactive properties of t18:1 and CLA isomers on primary bovine preadipocytes in terms of fat accumulation, fatty acid profiles,

adipogenic and lipogenic gene expression, and 2) compare the adipogenic potential of intramuscular SVF and DFAT preadipocytes.

Materials and Methods

Isolation of Stromal Vascular Fraction and Mature Adipocytes from Bovine Intermuscular Adipose Tissue

Intramuscular fat-derived stromal vascular fraction and mature adipocytes were collected from the brisket of three Angus heifers raised at the University of California Davis. Heifers were killed with a captive bolt stunner followed by exsanguination. Then, approximately 500 g of brisket tissue containing visible marbling fat was dissected and immediately placed in a cold DMEM with 1X antibiotics/antimycotics and transported to the laboratory. Information on the heifers is provided in Table 1.

Intramuscular fat-derived stromal vascular fraction (SVF) was isolated in a sterile biological safety cabinet using a method described by Mehta et al. (2019) with slight modifications. Briefly, about 5 g of intramuscular adipose tissue in perimysium was dissected from brisket and digested in 25 mL of DMEM containing 1X antibiotics/antimycotics and 200 unit/mL type II collagenase (Sigma Aldrich, Missouri, SL, USA) for an hour in a shaking incubator at 37℃ and 150 rpm, with constant inversion every 15 minutes. After digestion, tissue lysate was filtered through a 100µm sieve (Corning® Inc, Corning, NY, USA) and centrifuged at 500 x g for 5 minutes. The resulting SVF pellet was washed with DMEM 2 times and seeded in a 75cm^2 culture flask (VWR International, Radnor, PA, USA) with 8mL of culture media [DMEM with 10% fetal bovine serum (FBS) and 2X antibiotics/antimycotics] and incubated in a Forma 3110 CO2 Water Jacketed Incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37℃ with 5% CO2. Adhering SVF was expanded until 70~80% confluence and subcultured into 3

new 75cm^2 flasks. Resulting passage 1 SVFs were expanded until passage 3 to get enough SVF stock for experiments. Stock SVF was cryopreserved with the recovery media (Gibco, Thermo Fisher Scientific Waltham, MA, USA) in the vapor phase of liquid Nitrogen. DMEM, FBS, and antibiotics/antimycotics were all purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Preparation of Intramuscular Fat Derived Dedifferentiated Fat (DFAT) Cells

After the tissue lysate was centrifuged, the top layer containing mature adipocytes was collected and seeded in a 75cm^2 culture flask with a full load of culture media. Floating mature adipocytes were attached to the ceiling of the culture flask and incubated in the CO2 incubator at 37℃ with 5% CO2 for two weeks to dedifferentiate (Matsumoto et al., 2008). After mature adipocytes were dedifferentiated (i.e. mature adipocyte-derived DFAT cells), they were expanded until 70~80% confluence and then subcultured into three new flasks. The resulting passage 1 DFAT cells from each animal were mixed and expanded until passage 3 to prepare enough DFAT stocks for downstream experiments. Stock DFAT cells were cryopreserved with the recovery media and stored in the vapor phase of liquid Nitrogen.

Fatty acid-bovine serum albumin conjugation

To emulate in-vivo fatty acid transport in bovine plasma, free fatty acids (FFA) were conjugated with fatty-acid-free bovine serum albumin (Sigma Aldrich, Missouri, SL, USA) according to Evans et al. (2002). Briefly, 10mg/mL fatty acid stock solution was prepared in hexane. The volume of fatty acid stock required for 10mL of 4mM FFA-BSA solution was dried under N2 gas and dissolved in 1mL of 0.1M Potassium Hydroxide (KOH) solution. FFA-KOH solution was incubated in a heat block at 50℃ for 10 minutes with frequent inversions. FFA-KOH solution was then mixed with 7.5% BSA solution, incubated for 3 hours at room

temperature for conjugation, and stored at 4℃ overnight. The next day, the FFA-BSA solution was mixed with an additional 1 mL of 7.5% BSA solution and was divided into 1mL aliquot at -20℃ for future use. Pure free fatty acids (>98% purity) were purchased from Larodan (Solna, Sweden), Nu-Chek Prep Inc. (Elysian, MN, USA), and Life Lipids (San Diego, CA, USA).

Cell Viability Test

Before the experiments, the viability of cells against the fatty acid treatments (t10, c12-CLA, c9, t11-CLA, t10-18:1, and t11-18:1) was tested using CellTiter 96® AQueous One Solution (Promega, Madison, WI, USA). Briefly, DFAT and SVF cells were seeded in 96 well plates (1000 cells / well) and incubated for 24 hours with 100 µL of growth media containing fatty acid treatments at 4 different concentrations (0, 50, 100, and 200 µM). After the treatment period, 20 µL of CellTiter 96® AQueous One Solution was added to each well, and cells were incubated for 2 hours at 37 °C in 5% CO2; the absorbance at 490nm wavelength was recorded using a BioTek Synergy HT microplate reader (BioTek, Charlotte, VT, USA).

Adipogenic Differentiation Protocol

Passage 3 SVF and DFAT preadipocytes were thawed and seeded at 10^4 cells/cm^2 regardless of culture dish type. Cells were monitored every day until 100% confluence and incubated extra 24 hours after confluence for growth arrest. DFAT cells took 1-2 more days to reach 100% confluence than SVF cells. The adipogenic differentiation protocol was adopted by Kadegowda et al. (2013) with slight modifications. Cells were differentiated with differentiation medium (DMEM with 5% FBS, 2X Ab/Am, 0.25 µM Dex, 0.5mM IBMX, 5µM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate, and FFA-BSA test treatments) for 2 days. Then, the differentiation medium was replaced with maturation medium (DMEM with 5% FBS, 2X Ab/Am, 5uM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate, and FFA-BSA test

treatments) for 4 days. FFA treatments included 10μM of either t10, c12-CLA, c9, t11-CLA, t10-18:1, and t11-18:1. Control cell cultures received an equal volume of 7.5% BSA solution (BSA vehicle). At day 6 post-differentiation, cells were harvested for analysis.

Fatty acid analysis

Differentiated SVF and DFAT cells in 6-well plates (9.6cm^2 / well) were harvested with 0.25% trypsin-EDTA (Gibco, Thermo Fisher, Waltham, MA, USA), transferred into 10mL glass vials, rinsed with PBS, and then freeze-dried for 48 hours. Freeze-dried samples were then directly methylated using 0.5M sodium methoxide solution. Briefly, 0.05mg of internal standard fatty acid (cis10 17:1) in hexane and 2 mL of 0.5M sodium methoxide solution were added to the freeze-dried sample. Samples were incubated in a heat block at 50℃ for 10 minutes, with vortexing every 5 minutes. The resulting fatty acid methyl-ether (FAME) was dried under N2 gas, weighed, diluted by hexane up to 2mg/mL, and analyzed using a Trace 1310 Gas Chromatograph equipped with a flame-ionization detector (GC-FID, Thermo Fisher Scientific). Each sample was analyzed twice by GC using a 175°C plateau temperature program. The FAME was quantified using chromatographic peak area and internal standard-based calculations.

Oil Red O staining and lipid quantification

Differentiated SVF and DFAT cells in 24-well plates (1.9cm^2) were stained with Oil Red O dye using Lipid (Oil Red O) Staining Kit (Sigma Aldrich, Missouri, SL, USA) under manufacturer's instruction to visualize the lipid droplet and calculate the relative lipid accumulation. Oil Red O stock solution was prepared by dissolving powdered Oil Red O dye in isopropanol (3mg/mL) and filtering it with Whatman No.1 filter paper.

Briefly, differentiated cells at day 6 were fixed with 500 µL 10% formalin solution for 45 minutes, washed with 500 µL distilled water 2 times, and washed with 500 µL 60% isopropanol for 5 minutes. The fixed cells were stained with filtered 200 µL Oil Red O working solution (3 Oil Red O Stock Solution (60mg Oil Red O / 20mL isopropanol): 2 distilled water) for 15 minutes. Stained cells were washed with distilled water 5 times. Stained cells were observed with EVOS® FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA, USA). After getting images, stained cells were dried to evaporate the water and Oil Red O on lipid droplets was eluted to 100% isopropanol. OD at 492nm wavelength of the eluted Oil Red O in isopropanol was measured by the microplate reader to quantify the relative lipid accumulation in differentiated cells.

GPDH activity assay

Differentiated SVF and DFAT cells in 24-well plates (1.9cm^2) at day 6 of differentiation were assayed for GAPDH activity assay using GAPDH Activity Assay Kit (Sigma Aldrich, Missouri, SL, USA). Briefly, differentiated cells harvested in 200uL of GAPDH assay buffer were homogenized, kept on ice for 10 minutes, and centrifuged at 10000 x g for 5 minutes at 4˚. 25uL of centrifuged supernatant containing active GAPDH were mixed with Master Reaction Mix (GAPDH assay buffer, GAPDH developer, and GAPDH substrate) and incubated in the microplate reader in kinetic mode for 10-60 minutes at 37℃ to measure absorbance at 450nm. The amount of NADH produced was measured by a standard curve generated by wells containing 0 (blank), 2.5, 5.0, 7.5, 10, and 12.5 nmole NADH. The GADPH activity was calculated by following the equation. Two-time points (5 and 15 minutes) were chosen to calculate GAPDH activity per minute. GPDH activity was normalized to the total mg of protein

of a sample measured by Pierce™ BCA Protein Assay Kits (Pierce, Thermo Fisher Scientific, Waltham, MA, USA).

$$GPDH\ activity\ \left(\frac{nmol}{min * mL * mg\ Protein}\right) = \left(\frac{Amount\ of\ NADH\ generated * 2\ (Sample\ Dilution\ Factor)}{30\ (Time) * 25uL\ (Sample\ Volume) * mg\ Protein}\right.$$

Real-time RT-qPCR

Differentiated cells at day 6 were harvested for mRNA expression analysis of adipogenic and lipogenic genes. MRNA from differentiated cells were extracted using Invitrogen RNA Invitrogen™ PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions with minor modifications. The RNA concentration was measured by a Nano-drop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The quality of mRNA was assessed as a ratio of 260:280 absorbance at 10mm wavelength (all samples had a ratio higher than 1.6). The first strand cDNA of each sample was synthesized from 500ng of mRNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA). Samples were mixed with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and relevant primer pairs to determine the threshold cycle (Ct) by an Applied Biosystems MiniAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences for the internal control genes (EIF3K, GAPDH, and UXT) and target genes including PPARγ, C/EBPα, ACC, SCD-1, FAS, and CPT1B are listed in Table 1. The amplification efficiency for each primer pair was calculated from the slope of the standard curve generated with serial dilutions of a pooled cDNA sample using the formula ($E = 10^{(-1/slope)}$). The amplification efficiencies were between 90 and 105% for all primer pairs used in this study. Relative mRNA expression of target genes was

calculated using the ΔCt method with EIF3K, GAPDH, and UXT as the internal control genes. Each 96-well qPCR plate was set up to include reactions for both the target gene and the internal control gene for each cDNA sample. Target gene cycle threshold (Ct) values were normalized to that of EIF3K using $2^{-\Delta Ct}$ where ΔCt = Ct target gene – average Ct of EIF3K, GAPDH, and UXT (Riedel et al., 2014). Statistical analysis was performed on $2^{-\Delta Ct}$ data and the results were expressed as fold change relative to the BSA control.

Statistical Analysis

Data were analyzed using the mixed model procedure of SAS (v 9.3; SAS Institute, Cary, IN) with cell type (DFAT, SVF) and treatment (fatty acids) as the main effects. The model also tested two-way interaction effects between cell type and treatment. Differences between means were considered to be significant at $P < 0.05$ using the Tukey−Kramer multiple comparison test. Data are expressed as means ± standard error.

Results

Cytotoxicity of BSA-FFA conjugates

To check the cytotoxic concentration of fatty acid treatments, we exposed DFAT and SVF cells to DMEM containing 0, 50, 100, and 200 μM of fatty acid treatments for 48 hrs. There were no significant decreases ($P > 0.05$) in the cell count of both DFAT and SVF cells across all concentrations after incubation, as measured by Promega Aqueous One solution (Figure 1).

DFAT-derived preadipocytes have a greater adipogenic potential to SVF-derived adipocytes

To compare the adipogenic potential of DFAT and SVF cells, we exposed them to basal differentiation medium followed by maturation media. There was no difference ($P > 0.05$) in lipid accumulation between mature adipocytes derived from SVF and DFAT, as measured by Oil

Red O staining even though 100% confluent DFAT cells were less dense than SVF cells (Figure 2A). Furthermore, DFAT-derived mature adipocytes appeared to have larger and rounder lipid droplets than that of SVF-derived adipocytes (Figure 2B; Appendix Figure 2). It is noteworthy that the extent of lipid accumulation in both cell types was much higher when the differentiation medium was supplemented with fatty acids (Table 2; Figure 4).

The mRNA expression of PPARγ, the master regulator of adipogenesis, tended ($P = 0.06$) to increase after differentiation in both SVF and DFAT cells (Figure 3A). DFAT-derived mature adipocytes had a higher ($P = 0.004$) expression of C/EBPα, another key regulator of adipogenesis, compared to SVF cells (Figure 3B). However, level of C/EBPα mRNA expression stayed unchanged in both SVF and DFAT-derived adipocytes in response to the differentiation medium.

t10-18:1 did not promote lipid accumulation in DFAT- and SVF- derived adipocytes

The effect on lipid accumulation of 5, 10, 20 μM of fatty acid treatments (t10-18:1, t11-18:1, c9, t11-CLA, and t10, c12 CLA) was analyzed with Oil Red O relative lipid quantification (Figure 5). In both 5 and 10 μM fatty acid treated DFAT and SVF, t10-18:1 and t10, c12 CLA showed similar lipid accumulation compared to the BSA controls, while t11-18:1 and c9, t11-CLA exhibited significantly higher ($P < 0.05$) lipid content than BSA controls. When cultured with 20 μM of the fatty acid treatments, t10-18:1 had similar lipid content to that of BSA controls, while t11-18:1, c9, t11-CLA, and t10, c12-CLA exhibited significantly higher lipid accumulation compared to the BSA controls in both DFAT and SVF cells (Figure 5C).

t10-18:1 differently impacted GPDH activity in DFAT- versus SVF- derived adipocytes

To further investigate the effect of t-10 18:1, GPDH activities of both differentiated DFAT and SVF cells were analyzed. We chose 10 μM fatty acid treatment for GPDH activity assay and gene expression analysis because it is within the physiological level of t-18:1 found in the plasma of feedlot beef cattle. Moreover, both t10-18:1 and t10, c12-CLA at 10 μM concentration effectively demonstrated the reduction in lipid accumulation as evidenced by Oil Red O-based lipid quantification. In DFAT-derived adipocytes, the fatty acid treatments did not significantly ($P > 0.05$) affect GPDH activities, but t10, c12-CLA numerically had the lowest GPDH activity (Figure 6). In SVF-derived adipocytes, all fatty acid treatments showed higher ($P < 0.05$) GPDH activities than the BSA control, while t10-18:1 had a significantly lower ($P < 0.05$) GPDH activity compared to t11-18:1 and c9, t11-CLA treatments.

t10, c12-CLA but not t10-18:1 reduced the expression of lipogenic genes in both DFAT and SVF cells

In both DFAT and SVF-derived mature adipocytes, fatty acid treatments did not affect PPARγ expression compared to BSA control. Similarly, SREBP expression was not affected ($P > 0.05$) by any of the fatty acid treatments, with DFAT adipocytes showing higher expression of SREBP than SVF adipocytes.

In both cell models, t10, c12-CLA, which is known as a potent inhibitor of *de novo* lipogenesis, reduced ($P < 0.05$) the expression of lipogenic genes (SCD-1, FAS, and ACC) to the level of BSA controls (Figure 7CDE). Cells treated with t10-18:1, t11-18:1 or c9, t11-CLA had a higher ($P < 0.05$) expression of SCD-1 compared to BSA controls, in both DFAT and SVF models. Moreover, DFAT adipocytes exhibited higher ($P < 0.05$) SCD-1 expression than SVF adipocytes. There were no significant differences between the two cell types in terms of FAS and

ACC expression, but t10-18:1 reduced the expression of ACC comparable to BSA controls in both cell types. Furthermore, t10-18:1 decreased expression of FAS to the level of BSA control and t10, c12-CLA in DFAT cells, but it did not impact FAS expression in SVF cells.

DFAT-derived lipid droplets contained higher concentrations of supplemented fatty acids than SVF-derived lipid droplets

The effects of 10 μM fatty acid treatments on fatty acid profiles of DFAT- and SVF-derived lipids are presented in Table 2. In DFAT adipocytes, c9, t11-CLA, and t10, c12-CLA increased ($P < 0.05$) the proportion of saturated fatty acids (SFA) compared to t10-18:1, t11-18:1, and BSA control. In SVF, c9, t11-CLA ($P < 0.05$) increased the proportion of SFA compared to t10-18:1, t10, c12 CLA, and BSA control, while t11-18:1 decreased the SFA content compared to other fatty acid treatments and the BSA control. In both DFAT and SVF adipocytes, the content of cis monounsaturated fatty acids (cis-MUFA) was the highest ($P < 0.05$) in BSA controls, and there was no significant difference among the fatty acid-treated adipocytes. The content of polyunsaturated fatty acids (PUFA) was higher in t10-18:1 treated DFAT and SVF adipocytes than in other fatty acid treatments, with SVF having a higher PUFA content than DFAT adipocytes. Overall, the uptake of supplemented fatty acids was ~2 times higher in DFAT than that in SVF adipocytes, as evidenced by total trans fatty acid (TFA) content in adipocytes. In DFAT adipocytes, the t-18:1 treatments (t10- and t11-18:1) had the highest accumulation followed by CLA treatments (c9, t11-CLA and t10, c12-CLA). In SVF adipocytes, t11-18:1 and t10, c12-CLA had the highest accumulations followed by t10-18:1 and c9-t11-CLA.

Discussion

Most studies of the regulation of bovine intramuscular adipogenesis have been conducted in SVF-derived preadipocyte cultures. A few studies have investigated the adipogenesis of

bovine DFAT cells (Wei et al., 2012; Wei et al., 2013). However, to our knowledge, no published study compares adipogenesis in primary bovine SVF and DFAT cells. Thus, we assessed the mRNA expression of two key markers of adipogenic differentiation (C/EBPβ and PPAR γ) and the lipid content (Oil Red O quantitation) before (undifferentiated preadipocytes) and 6 days after differentiation induction (differentiated/mature adipocytes) to compare the adipogenic potential of intramuscular DFAT and SVF cells. The expression of C/EBP α, which is known to be responsible for early phases of adipogenesis, was not different between preadipocytes and mature adipocytes within the two cell types. Conversely, C/EBP α expression was significantly higher in DFAT compared to SVF cells, suggesting greater adipogenic potential in DFAT cells. Consistent with this finding, the extent of lipid accumulation after culturing with differentiation/maturation media tended to be higher in DFAT compared to SVF cells.

The greater adipogenic response of DFAT cells could be due to the higher percentage of mesenchymal adipogenic progenitors compared to SVF cells (Matsumoto et al., 2008; Shen et al., 2011; Wei et al., 2012). Flow cytometry done by Matsumoto et al. (2008) showed that human DFAT cells contain a more homogeneous mesenchymal stem cell population than SVF which also contains other cell types such as smooth muscle cells, lymphocytes, monocytes, and endothelial cells. Moreover, other studies with human adipocytes have consistently shown that DFAT cells exert more adipogenic and lipogenic capacitiy than SVF (Watson et al., 2014; Fujii et al., 2012).

It is notable to mention that compared to SVF-derived adipocytes, DFAT-derived adipocytes had a higher content of supplemented fatty acids . This suggests that DFAT cells have a higher fatty acid uptake capacity than the SVF cells, which could be in part related to higher

expression of fatty acid binding protein 4 (FABP4) in DFAT-derived mature adipocytes (Beloor et al., 2010; Fujii et al. (2012). However, FABP expression was not assessed in the present study.

The second main objective of the present study was to test whether t10-18:1 would have similar anti-lipogenic effects to that of t10, c12-CLA in SVF and DFAT cell models. As t10, c12 CLA has consistently been identified as a potent inhibitor of adipogenesis and lipogenesis in 3T3 cells (Kang et al., 2003; Vahmani et al., 2016), we expected the lowest lipid accumulation in t10, c12-CLA treated cells. Interestingly, 5, 10, and 20 μM of t10, c12 CLA supplement increased lipid accumulation in both DFAT- and SVF-derived mature adipocytes (Figure 5). Consistent with our findings, Kadegowda et al. (2013) showed that culturing bovine adipose-derived SVF cells with t10, c12-CLA increased cellular lipid content, which was in part attributed to uptake of supplemented t10, c12-CLA by adipocytes. Furthermore, culturing with t10, c12-CLA depressed SCD expression (Figure 8) and c9-16:1/16:0 and c9-18:1/18:0 ratios (Table 2) which was consistent with Kadegowda et al. (2013). In fact, a number of *in vivo* and *in vitro* studies the have shown that the anti-lipogenic effects of t10, c12-CLA are mainly related to its inhibitory effects on desaturation rather than *de novo* fatty acid synthesis (Chung et al., 2006;).

Strikingly, we observed that t10-18:1 treated SVF and DFAT cells had the lowest lipid accumulation (i.e. Oil Red O stained lipid droplets) among all fatty acid-treated cells at any tested dose (5, 10, or 20 μM). Consistent with the Oil Red O results, GPDH activity, which is a marker of late-stage of adipogenesis, was lower in t10-18:1 treated SVF cells compared to other fatty acid-treated SVF cells. Given the cellular incorporation level of supplemented fatty acids (as evidenced by the total TFA content of adipocytes) was comparable across fatty acid treatments (Table 2), it is unlikely that reduced lipid accumulation in t10-18:1 treated cells was due to lower uptake/accumulation of t10-18:1 by the cells.

Conversely, both t10-18:1 and t10, c12 CLA reduced ACC and FAS mRNA expression compared to t11-18:1 and c9, t11-CLA treatments in DFAT cells. The t10, c12 CLA effects on ACC and FAS gene expression is consistent with published studies in primary bovine adipocytes (Chung et al., 2006; Kadegowda et al., 2013; Choi et al., 2014). However, the fact that t10-18:1 treated cells had a lower lipid content than t10, c12-CLA treated cells in the present study (Figure 5) cannot be explained by the lipogenic gene expression data (Figure 7). The only published cell culture study on t10-18:1 effects used differentiated 3T3-L1 cells as a model (Park et al., 2004). This study showed that t-10 18:1 stimulates glycerol release in 3T3-L1 cells. Thus, it is possible that the lower lipid content of t10-18:1 treated cells in our study was related to increased lipolysis by t10-18:1, which deserves further investigation.

Conclusion

In summary, DFAT cells appear to have a greater adipogenic and fatty acid uptake capacity compared to SVF cells. However, both cell types showed a similar lipogenic response (lipogenic gene expression and lipid accumulation) to the fatty acid treatments. Thus, both cell types are valid models to test/screen for potential anti-lipogenic of natural compounds including rumen-derived bioactive fatty acids.

Among the fatty acid tested in the present study, only t10-18:1 inhibited lipid accumulation in primary intramuscular bovine adipocytes. Despite reducing lipid accumulation in cultured adipocytes, t10-18:1 did not affect lipogenic gene expression or GPDH activity. Conversely, t10, c12-18:2 significantly inhibited desaturation activity while increasing lipid accumulation in cultured bovine adipocytes. More studies are needed to elucidate the mechanisms by which t10-18:1 and t10, c12-18:2 may affect marbling development in beef

cattle, including their potential synergistic effects on adipogenous and lipogenesis in bovine adipocytes.

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Tables

Table 1. Primer list used for RT-qPCR.

Gene[1]	Forward (5'-3')	Reverse (3'-5')	Source
PPARγ	ATCTGCTGCAAGCCTTGGA	TGGAGCAGCTTGGCAAAGA	Choi et al., 2014.
ACC1	AGCTGAATTTTCGCAGCAAT	GGTTTTCTCCCCAGGAAAAG	Duckett et al., 2009.
FAS	GCATCGCTGGCTACTCCTAC	GTGTAGGCCATCACGAAGGT	Duckett et al., 2009.
C/EBPa	TGGACAAGAACAGCAACGAG	GGTCATTGTCACTGGTCAGC	Duckett et al., 2009.
SCD1	CGCCCTTATGACAAGACCAT	TGGCAGCCTTGGATACTTTC	Yue et al., 2018.
GAPDH	GATGCTGGTGCTGAGTATGT	GCAGAAGGTGCAGAGATGAT	Zhang et al., 2017
UXT	CAGCTGGCCAAATACCTTCAA	GTGTCTGGGACCACTGTGTCAA	Yanting et al., 2018
EIF 3K	CTTCTGGCAAGCCCTGGATG	TAGCTGGCTGTCTGTCAGATCCC	Yanting et al., 2018

[1] PPARγ = peroxisome proliferator activated receptor gamma; ACC1 = acetyl-coA carboxylase 1; FAS = fatty acid synthase; C/EBPa = CCAAT enhancer binding protein alpha; SCD1 = stearoyl-CoA desaturase 1; GAPDH glyceraldehyde-3-phosphate dehydrogenase; UXT = ubiquitously expressed transcript; EIF 3K = eukaryotic-transcription initiation factor 3 subunit K

Table 2. Fatty acid composition (% of total fatty acids) of bovine DFAT[1]- and SVF[2]-derived adipocyte cultures supplemented with 0 (BSA control) or 10μM of t10-18:1, t11-18:1 (TVA), c9, t11-CLA or t10, c12-CLA.

Cell Type	DFAT-derived adipocytes					SVF-derived adipocytes					Effect (P-value)[3]		
Treatment / Fatty acid[4]	BSA Control	t10-18:1	t11-18:1	c9, t11-CLA	t10, c12-CLA	BSA Control	t10-18:1	t11-18:1	c9, t11-CLA	t10, c12-CLA	Cell	Treatment	Cell x Treatment
16:0	$18.62^{C}\pm0.566$	$13.85^{E}\pm0.19$	$15.36^{DE}\pm0.332$	$16.61^{CD}\pm1.07$	$15.20^{DE}\pm0.23$	$18.88^{C}\pm1.887$	$21.78^{B}\pm2.063$	$18.56^{C}\pm1.379$	$25.72^{A}\pm1.153$	$21.24^{B}\pm2.428$	<.0001	0.0005	<.0001
c9-16:1	$0.062^{BC}\pm0.017$	$0.45^{CD}\pm0.071$	$0.50^{CD}\pm0.094$	$0.87^{A}\pm0.304$	$0.47^{CD}\pm0.202$	$0.81^{AB}\pm0.211$	$0.28^{D}\pm0.064$	$0.39^{CD}\pm0.087$	$0.42^{CD}\pm0.034$	$0.30^{D}\pm0.05$	0.0120	0.0008	0.0185
t8-16:1	0.00^{C}	$2.02^{A}\pm0.107$	0.000^{C}	0.000^{C}	0.000^{C}	0.000^{C}	$1.13^{B}\pm0.349$	0.000^{C}	0.000^{C}	0.000^{C}	<.0001	<.0001	<.0001
t9-16:1	0.00^{C}	0.000^{C}	$5.24^{A}\pm0.266$	0.000^{C}	0.000^{C}	0.000^{C}	0.000^{C}	$4.13^{B}\pm0.552$	0.000^{C}	0.000^{C}	<.0001	<.0001	<.0001
18:0	$17.62^{A}\pm0.546$	$10.10^{E}\pm0.447$	$8.90^{F}\pm0.633$	$12.45^{CD}\pm0.10$	$12.66^{C}\pm1.000$	$16.90^{A}\pm0.585$	$12.25^{CD}\pm2.281$	$11.58^{D}\pm0.455$	$14.57^{B}\pm0.312$	$14.95^{B}\pm0.452$	<.0001	<.0001	0.0003
c9-18:1	$15.77^{A}\pm0.712$	$10.58^{DE}\pm0.942$	$10.93^{CD}\pm0.392$	$10.53^{DE}\pm0.24$	$9.96^{E}\pm0.202$	$15.48^{A}\pm0.261$	$12.05^{B}\pm0.475$	$11.62^{BC}\pm0.157$	$10.11^{DE}\pm0.221$	$10.21^{DE}\pm0.66$	0.0761	<.0001	0.022
c11-18:1	$5.14^{A}\pm0.246$	$4.01^{B}\pm0.722$	$3.45^{C}\pm0.152$	$3.33^{C}\pm0.367$	$3.75^{C}\pm0.379$	$6.01^{A}\pm0.381$	$5.23^{B}\pm0.474$	$4.76^{C}\pm0.22$	$4.40^{C}\pm0.132$	$4.37^{C}\pm0.045$	<.0001	<.0001	0.4956
t10-18:1	0^{C}	$23.50^{A}\pm1.558$	0^{C}	0^{C}	0^{C}	0^{C}	$10.1247^{B}\pm0.53$	0^{C}	0^{C}	0^{C}	<.0001	<.0001	<.0001
t11-18:1	0^{C}	0^{C}	$19.68^{A}\pm1.354$	0^{C}	0^{C}	0^{C}	0^{C}	$10.02^{B}\pm0.136$	0^{C}	0^{C}	<.0001	<.0001	<.0001
18:2n-6	$4.28^{A}\pm0.112$	$3.00^{C}\pm0.29$	$3.25^{C}\pm0.38$	$2.90^{C}\pm0.207$	$3.27^{C}\pm0.06$	$3.73^{B}\pm0.209$	$4.06^{AB}\pm0.366$	$3.94^{AB}\pm0.099$	$3.76^{B}\pm0.139$	$4.17^{A}\pm0.116$	<.0001	0.0008	<.0001
20:3n-6	$2.92^{A}\pm0.477$	$2.33^{B}\pm0.053$	$2.15^{B}\pm0.242$	$1.96^{C}\pm0.255$	$1.84^{C}\pm0.124$	$3.82^{A}\pm0.306$	$3.12^{B}\pm0.125$	$3.22^{B}\pm0.162$	$2.37^{C}\pm0.16$	$2.31^{C}\pm0.296$	<.0001	<.0001	0.1431
20:4n-6	$11.99^{A}\pm0.563$	$9.59^{B}\pm0.424$	$9.00^{B}\pm0.594$	$6.17^{C}\pm0.283$	$7.19^{D}\pm0.438$	$15.35^{A}\pm0.945$	$13.47^{B}\pm1.092$	$13.56^{B}\pm0.525$	$9.72^{C}\pm0.242$	$11.75^{D}\pm0.917$	<.0001	<.0001	0.4002
22:5n-3	$3.50^{A}\pm0.41$	$3.26^{A}\pm0.379$	$3.47^{AB}\pm0.412$	$2.89^{AB}\pm0.236$	$3.27^{B}\pm0.281$	$5.63^{A}\pm0.341$	$5.08^{A}\pm0.553$	$5.50^{AB}\pm0.096$	$4.60^{AB}\pm0.527$	$5.17^{B}\pm0.598$	<.0001	0.0209	0.9152
22:6n-3	$2.14^{A}\pm0.113$	$2.03^{A}\pm0.219$	$2.35^{A}\pm0.668$	$1.83^{AB}\pm0.238$	$2.16^{B}\pm0.165$	$4.52^{A}\pm0.417$	$4.19^{A}\pm0.259$	$4.09^{A}\pm0.347$	$3.26^{AB}\pm0.028$	$3.71^{B}\pm0.444$	<.0001	0.0064	0.1121
c9, t11-CLA	0^{E}	0^{E}	$1.98^{C}\pm0.086$	$21.93^{A}\pm0.253$	0^{E}	0^{E}	0^{E}	$1.24^{D}\pm0.046$	$10.50^{B}\pm0.043$	0^{E}	<.0001	<.0001	<.0001
t10, c12CLA	0^{C}	0^{C}	0^{C}	0^{C}	$20.58^{A}\pm1.744$	0^{C}	0^{C}	0^{C}	0^{C}	$12.73^{B}\pm0.812$	<.0001	<.0001	<.0001
∑SFA	$37.83^{B}\pm0.065$	$25.40^{D}\pm0.531$	$25.41^{D}\pm0.936$	$30.85^{C}\pm1.427$	$29.29^{C}\pm1.216$	$37.1596^{B}\pm1.72$	$36.78^{B}\pm3.937$	$31.3123^{C}\pm1.02$	$41.93^{A}\pm1.155$	$37.61^{B}\pm2.93$	<.0001	<.0001	<.0001
∑cMUFA	$23.65^{A}\pm1.103$	$15.41^{D}\pm0.296$	$16.62^{CD}\pm0.676$	$16.55^{CD}\pm0.59$	$15.45^{CD}\pm0.27$	$25.31^{A}\pm0.53$	$19.27^{B}\pm1.206$	$18.93^{B}\pm0.229$	$20.02^{BC}\pm0.403$	$16.56^{CD}\pm0.66$	<.0001	<.0001	0.0012
∑PUFA	$28.33^{A}\pm0.877$	$22.72^{B}\pm1.143$	$23.22^{BC}\pm2.296$	$18.39^{C}\pm0.726$	$20.85^{D}\pm0.958$	$37.12^{A}\pm2.055$	$32.18^{B}\pm2.229$	$34.36^{BC}\pm1.39$	$27.24^{C}\pm1.035$	$31.20^{D}\pm2.781$	<.0001	<.0001	0.7094
∑TFA	0.00^{F}	$27.71^{A}\pm1.476$	$26.91^{A}\pm1.629$	$22.65^{B}\pm0.253$	$21.06^{B}\pm1.744$	0.00^{F}	$11.77^{DE}\pm0.73$	$15.40^{C}\pm0.583$	$10.82^{E}\pm0.043$	$13.03^{D}\pm0.812$	<.0001	<.0001	<.0001
16:1/16:0 ratio	$0.033^{BC}\pm0.000$	$0.033^{BC}\pm0.005$	$0.032^{BC}\pm0.005$	$0.052^{A}\pm0.015$	$0.03^{BC}\pm0.014$	$0.042^{AB}\pm0.008$	$0.013^{D}\pm0.003$	$0.021^{CD}\pm0.003$	$0.017^{D}\pm0.002$	$0.014^{D}\pm0.001$	<.0001	0.0054	0.0011
18:1/18:0 ratio	$0.89^{C}\pm0.029$	$1.05^{B}\pm0.089$	$1.23^{A}\pm0.045$	$0.85^{CD}\pm0.014$	$0.79^{D}\pm0.05$	$0.92^{C}\pm0.021$	$1.01^{B}\pm0.073$	$1.00^{B}\pm0.029$	$0.69^{E}\pm0.01$	$0.68^{E}\pm0.026$	<.0001	<.0001	0.0012

[1] Mature adipocyte-derived dedifferentiated fat cells (DFAT)

[2] Stromal Vascular Fraction (SVF)

[3] *P*-values for the effects of Cell (DFAT vs. SVF), Treatment (BSA-control, t10-18:1, t11-18:1, c9, t11-CLA, or t10, c12-CLA), and their interaction (Cell x Treatment)

[4] *c* = *cis*, *t* = *trans*; CLA: conjugated linoleic acid, ∑SFA: Sum of saturated fatty acids, ∑cMUFA: Sum of cis monounsaturated fatty acids, ∑PUFA: Sum of polyunsaturated fatty acids (n-6PUFA+n-3-PUFA), ∑TFA: Sum of fatty acids that contain at least one trans double bond (*t*16:1 + *t*18:1 + *c*,*t*-18:2 + *t*,*c*-18:2 + *t*20:1); 16:1/16:0 ratio: cis9-16:1/16:0 ratio or stearoyl-CoA desaturase (SCD)-16 index; cis9-18:1/18:0 ratio or stearoyl-CoA desaturase (SCD)-18 index.

[A-F] Within each row, means without a common superscript differ ($P < 0.05$; N = 3).

Figures

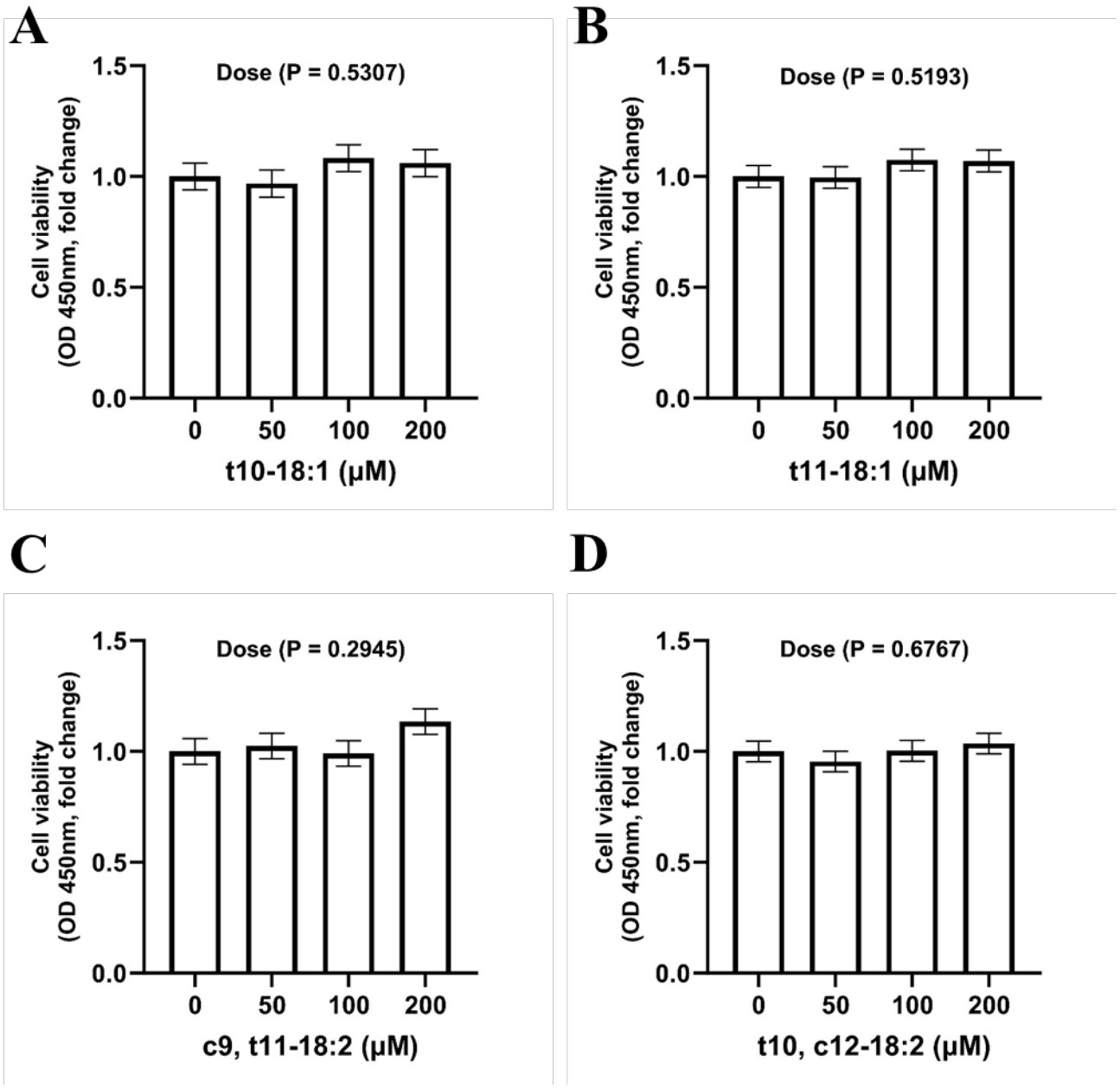

Figure 1. The effect of selected fatty acids on cell viability. The viability of dedifferentiated fat (DFAT)-derived preadipocytes treated with t10-18:1, t11-18:1, c9, t11-CLA, or t10, c12-CLA in 4 different doses [0, 50, 100, and 200 μM] was evaluated after 48-h incubation. The test medium was replaced after incubation with fresh DMEM containing 200μL of CellTiter 96® AQueous One Solution and incubated for 2 hours. After incubation, the absorbance at 450nm wavelength was measured by BioTek Synergy HT microplate reader. Data were analyzed using the mixed

model procedure of SAS and the statistical model included the main effects of doses (Dose). Values are expressed as mean ± standard error of the mean (N (wells) = 5).

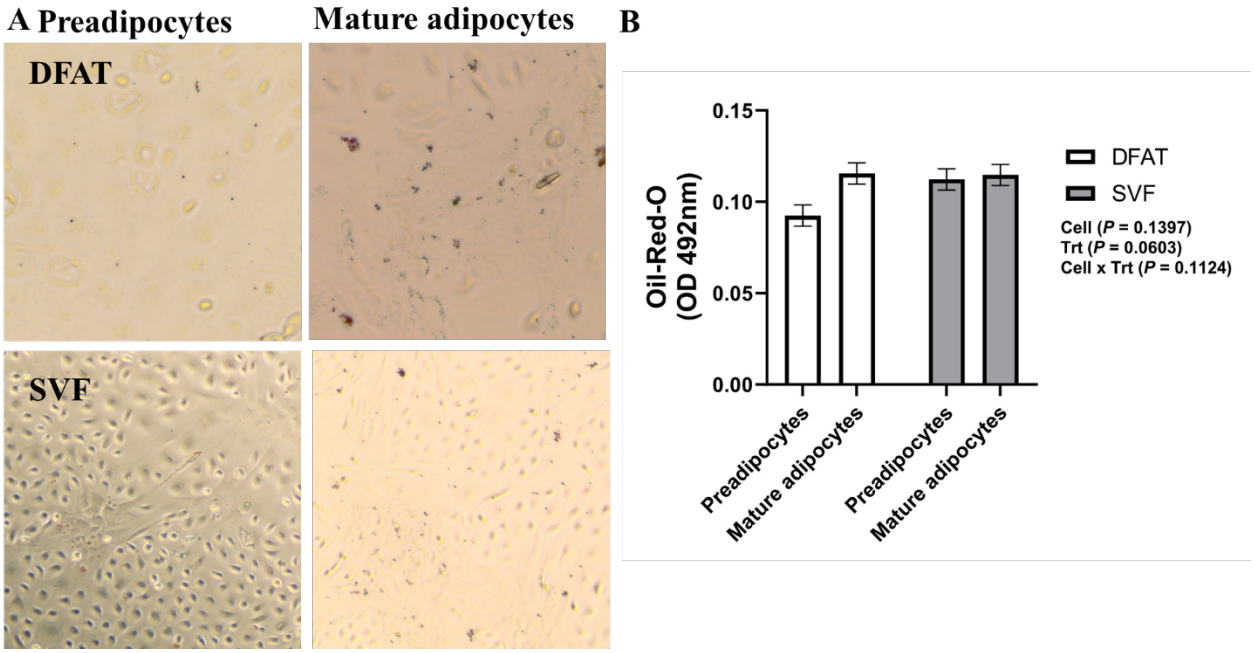

Figure 2. Microscopic morphologies of bovine preadipocytes and mature adipocytes derived from dedifferentiated fat cells (DFAT) or stromal vascular fraction (SVF), stained with Oil-red-O, and then observed with 40x magnification (A). The Oil-red-O stain was extracted in isopropanol and quantification by measuring absorbance at 492 nm (B). DFAT and SVF preadipocytes were maintained in the growth medium [DMEM with 5% fetal bovine serum (FBS) and 2X antibiotics/antimycotics] for 6 days post-confluency, followed by Oil-red-O staining. For mature adipocytes, SVF and DFAT preadipocytes were differentiated using a growth medium supplemented with 0.25 µM Dex, 0.5mM IBMX, 5µM Troglitazone, 2.5 µg/mL bovine insulin, and 10mM acetate. After 2 days, the differentiation medium was replaced with a maturation medium (DMEM with 5% FBS, 2X antibiotics/antimycotics, 5uM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate) for 4 days. The mature adipocytes (6 days post-differentiation) were used for Oil-red-O staining and quantification. Data were analyzed using the mixed model procedure of SAS and the statistical model included main effects of cell type (Cell), treatment (TRT), and their interaction (Cell× Med). Values are expressed as mean ± standard error of the mean (N (wells) = 5).

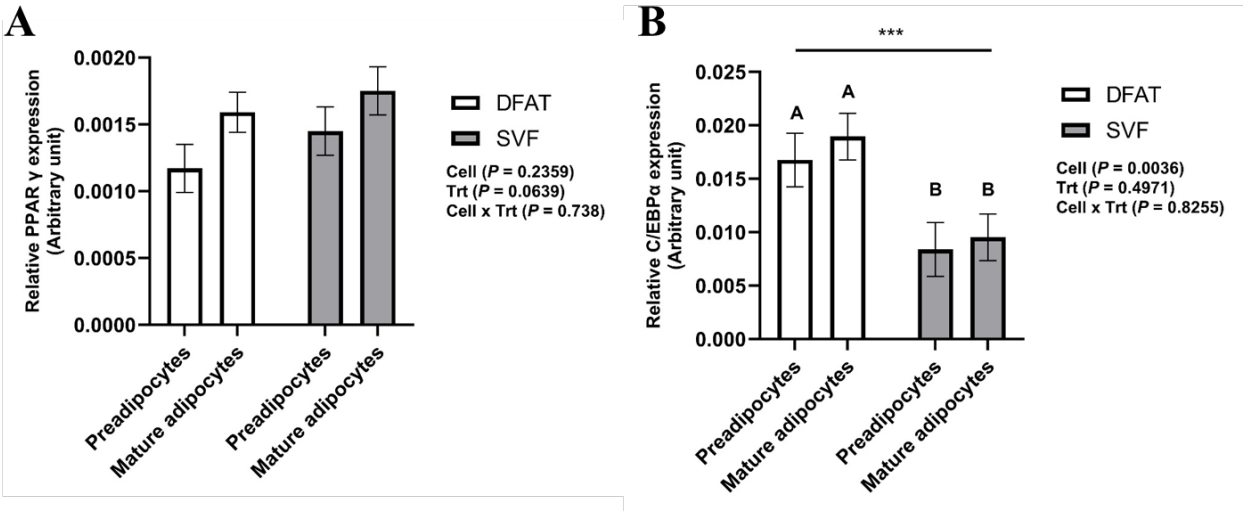

Figure 3. Relative mRNA expression of peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein alpha (C/EBPα), and Stearoyl-coenzyme A desaturase 1 (SCD1) in bovine preadipocytes and mature adipocytes derived from dedifferentiated fat cells (DFAT) or stromal vascular fraction (SVF). DFAT and SVF preadipocytes were maintained in the growth medium [DMEM with 5% fetal bovine serum (FBS) and 2X antibiotics/antimycotics] for 6 days post-confluency, followed by mRNA extraction for qPCR analysis. For the mature adipocytes, SVF and DFAT preadipocytes were differentiated using a growth medium supplemented with 0.25 µM Dex, 0.5mM IBMX, 5µM Troglitazone, 2.5 µg/mL bovine insulin, and 10mM acetate. After 2 days, the differentiation medium was replaced with a maturation medium (DMEM with 5% FBS, 2X antibiotics/antimycotics, 5uM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate) for 4 days. The mature adipocytes (6 days post-differentiation) were used for qPCR analysis. Data were analyzed using the mixed model procedure of SAS and the statistical model included the main effects of cell type (Cell), treatment (TRT), and their interaction (Cell× TRT). Values are expressed as mean ± standard error of the mean (N (wells) = 5). ^{ABC} Means across the cell types with common superscripts are not different ($P > 0.05$). ^{abc} Means within a cell type with common superscripts are not different ($P > 0.05$). *** Refers to significant main effect of cell type ($P < 0.05$).

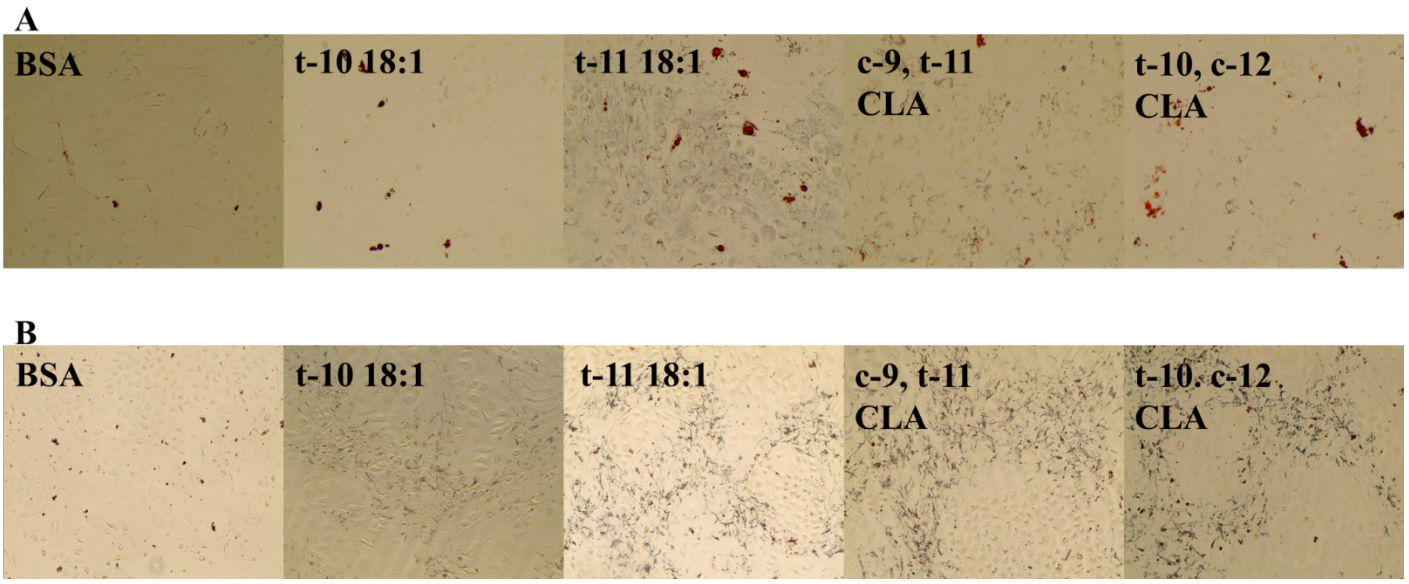

Figure 4. Microscopic morphologies of bovine mature adipocytes derived from (A) dedifferentiated fat cells (DFAT) or (B) stromal vascular fraction (SVF) differentiated and maturated with 10 µM of t10-18:1, t11-18:1, c9, t11-CLA, t10, c12-CLA, or with no added fatty acids (BSA control) in 10x magnification. SVF and DFAT preadipocytes were differentiated using a growth medium supplemented with 0.25 µM Dex, 0.5mM IBMX, 5µM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate, and 10 µM test treatments. After 2 days, the differentiation medium was replaced with a maturation medium (DMEM with 5% FBS, 2X antibiotics/antimycotics, 5uM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate, and 10 µM test treatments) for 4 days. Test treatments were supplemented in both differentiation and maturation medium. Data were analyzed using the mixed model procedure of SAS and the statistical model included main effects of cell type (Cell), treatment (TRT), and their interaction (Cell× TRT). Values are expressed as mean ± standard error of the mean (N (wells) = 5). ^{ABC} Means across the cell types with common superscripts are not different ($P > 0.05$). ^{abc} Means within a cell type with common superscripts are not different ($P > 0.05$). *** Refers to significant main effect of cell type ($P < 0.05$).

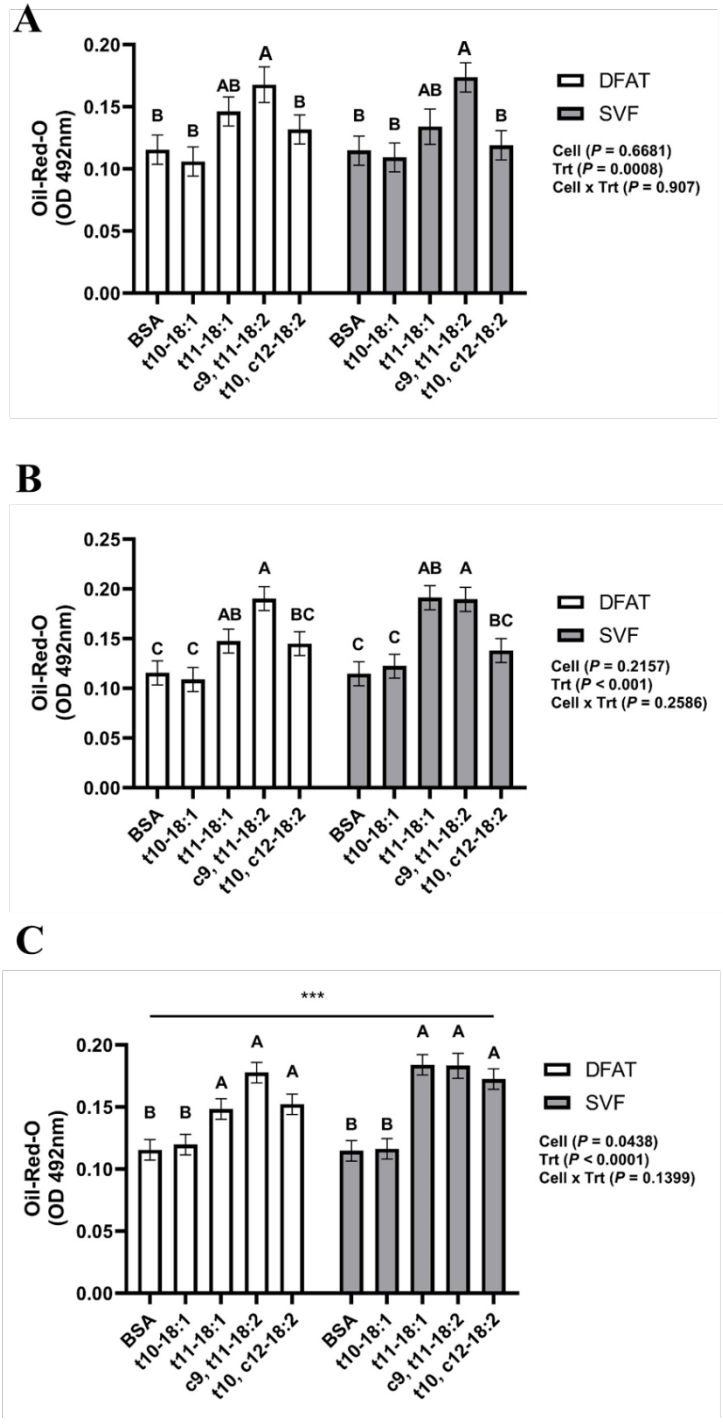

Figure 5. Effects of (A) 5, (B) 10, and (C) 20 µM of t10-18:1, t11-18:1, c9, t11-CLA, t10, c12-CLA, or with no added fatty acids (BSA control) on lipid accumulation in mature adipocytes measured by quantification of oil red O staining. SVF and DFAT preadipocytes were differentiated using a growth medium supplemented with 0.25 µM Dex, 0.5mM IBMX, 5µM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate, and test treatments. After 2 days, the

differentiation medium was replaced with a maturation medium (DMEM with 5% FBS, 2X antibiotics/antimycotics, 5uM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate, and test treatments) for 4 days. Oil-red-O stained the lipid droplets in mature adipocytes (6 days post differentiation) were dissolved in isopropanol and measured by BioTek Synergy HT microplate reader for lipid quantification. Data were analyzed using the mixed model procedure of SAS and the statistical model included main effects of cell type (Cell), treatment (TRT), and their interaction (Cell× TRT). Values are expressed as mean ± standard error of the mean (N (wells) = 5). ^{ABC} Means across the cell types with common superscripts are not different (P > 0.05). ^{abc} Means within a cell type with common superscripts are not different (P > 0.05). *** Refers to significant main effect of cell type (P < 0.05).

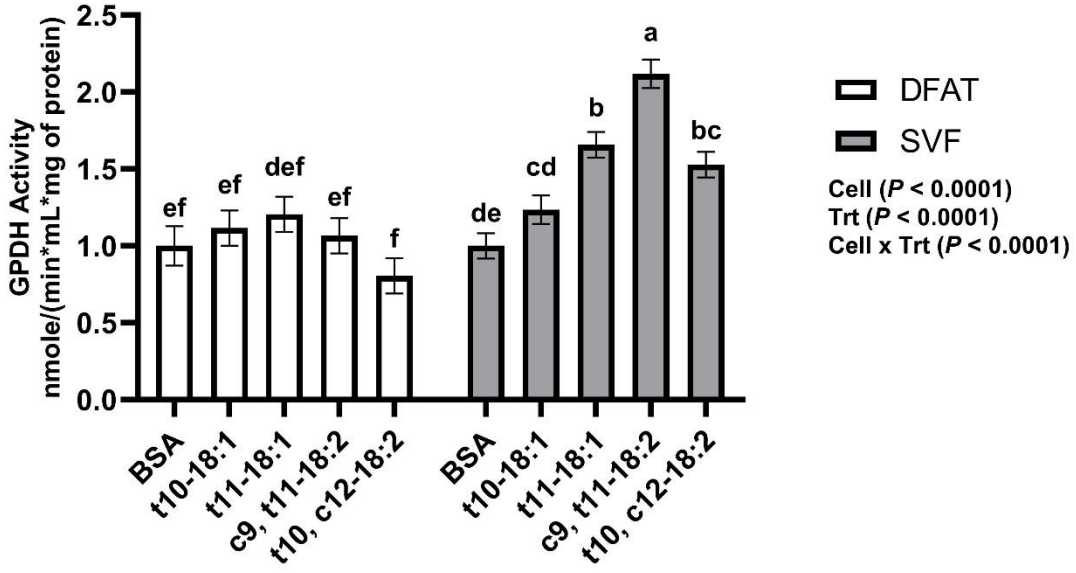

Figure 6. GPDH activities of DFAT- and SVF-derived preadipocytes differentiated and maturated with 10 µM t10-18:1, t11-18:1, c9, t11-CLA, t10, c12-CLA, or with no added fatty acids (BSA control). SVF and DFAT preadipocytes were differentiated using a growth medium supplemented with 0.25 µM Dex, 0.5mM IBMX, 5µM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate, and test treatments. After 2 days, the differentiation medium was replaced with a maturation medium (DMEM with 5% FBS, 2X antibiotics/antimycotics, 5uM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate, and test treatments) for 4 days. The mature adipocytes (6 days post differentiation) were used for GPDH activity assay. The GPDH activity measured was normalized to the amount of protein measured by the Pierce BCA kit [nmole of NAD/min]. The activity was expressed as the fold change relative to BSA control. Data were analyzed using the mixed model procedure of SAS and the statistical model included main effects of cell type (Cell), treatment (TRT), and their interaction (Cell× TRT). Values are expressed as mean ± standard error of the mean (N = 5). ^{ABC} Means across the cell types with common superscripts

are not different (P > 0.05). [abc] Means within a cell type with common superscripts are not different (P > 0.05). *** Means there was a significant difference across the cell types (P < 0.05).

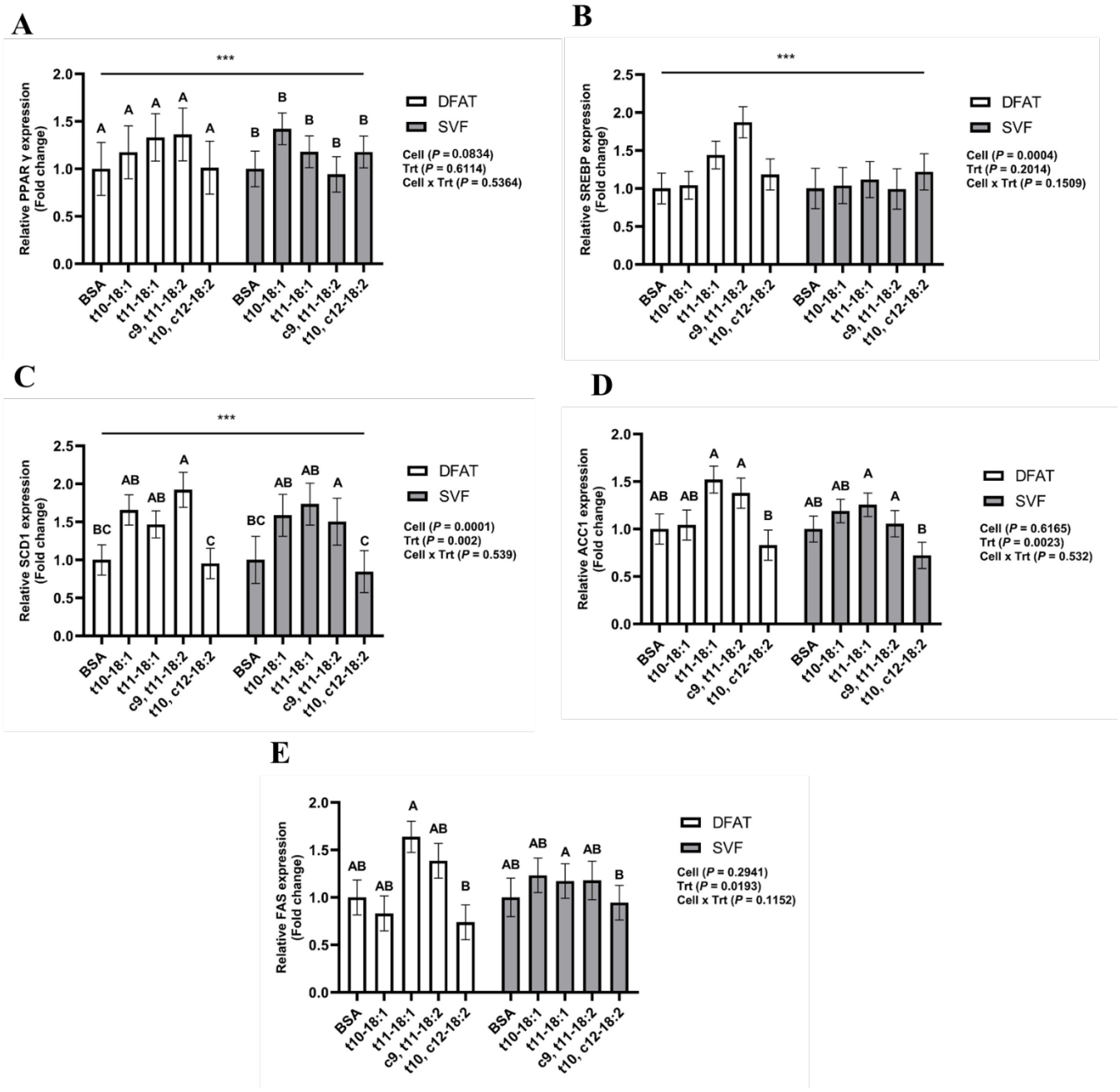

Figure 7. Relative mRNA expression of peroxisome proliferator-activated receptor gamma (PPARγ), sterol regulatory element binding protein (SREBP), Stearoyl-coenzyme A desaturase 1 (SCD1), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) in bovine mature adipocytes derived from dedifferentiated fat cells (DFAT) or stromal vascular fraction (SVF) differentiated and maturated with 10 μM t10-18:1, t11-18:1, c9, t11-CLA, t10, c12-CLA, or with

no added fatty acids (BSA control). SVF and DFAT preadipocytes were differentiated using a growth medium supplemented with 0.25 µM Dex, 0.5mM IBMX, 5µM Troglitazone, 2.5 µg/mL bovine insulin, 10 mM acetate, and 10 µM test treatments. After 2 days, the differentiation medium was replaced with a maturation medium (DMEM with 5% FBS, 2X antibiotics/antimycotics, 5uM Troglitazone, 2.5 µg/mL bovine insulin, 10mM acetate, and 10 µM test treatments) for 4 days. The mature adipocytes (6 days post-differentiation) were used for qPCR analysis. Data were analyzed using the mixed model procedure of SAS and the statistical model included main effects of cell type (Cell), treatment (TRT), and their interaction (Cell× TRT). Values are expressed as mean ± standard error of the mean (N = 5). ^{ABC} Means across the cell types with common superscripts are not different ($P > 0.05$). ^{abc} Means within a cell type with common superscripts are not different ($P > 0.05$). *** Refers to significant main effect of cell type ($P < 0.05$).

Appendix

Table 1. Information of donor animals and starter/transition/finish rations received

Ear Tag	Diet	Live Weight (lb)	Carcass Weight (lb)	Gender
1118	7 days fed alfalfa hay 30 days fed starter ration 14 days fed transition ration 123 days fed finish ration	1354	882	Heifer
J042	30 days fed alfalfa hay 120 days fed starter ration 14 days fed transition ration 60 days fed finish ration	1273	738	Heifer
1015	7 days fed alfalfa hay 30 days fed starter ration 14 days fed transition ration 123 days fed finish ration	1519	1030	Heifer

Ration	Ingredients	% of the diet (as-fed)
Starter	Rolled Corn	41.991%
	DDG	20.000%
	Fat	1.500%
	Molasses	8.000%
	Alfalfa Hay	15.000%
	Wheat Hay	12.000%
	Calcium Carbonate	0.820%
	Urea	0.350%
	Magnesium Oxide	<0.001%
	Rumensin	0.020%
	Beef Trace Salt	0.319%
Transition	Rolled Corn	51.116%
	DDG	20.000%
	Fat	2.000%
	Molasses	7.000%
	Alfalfa Hay	10.000%
	Wheat Hay	8.000%
	Calcium Carbonate	1.150%
	Urea	0.400%
	Magnesium Oxide	<0.000%
	Rumensin	0.015%
	Beef Trace Salt	0.319%
Finish	Rolled Corn	52.340%
	DDG	26.880%
	Wheat Hay	10.330%
	Water	5.000%
	Fat	2.750%
	Molasses	0.870%
	Calcium Carbonate	1.450%
	Salt	0.300%
	Magnesium Oxide	0.030%
	UMW Feedlot Premix	0.040%
	Rumensin	0.010%

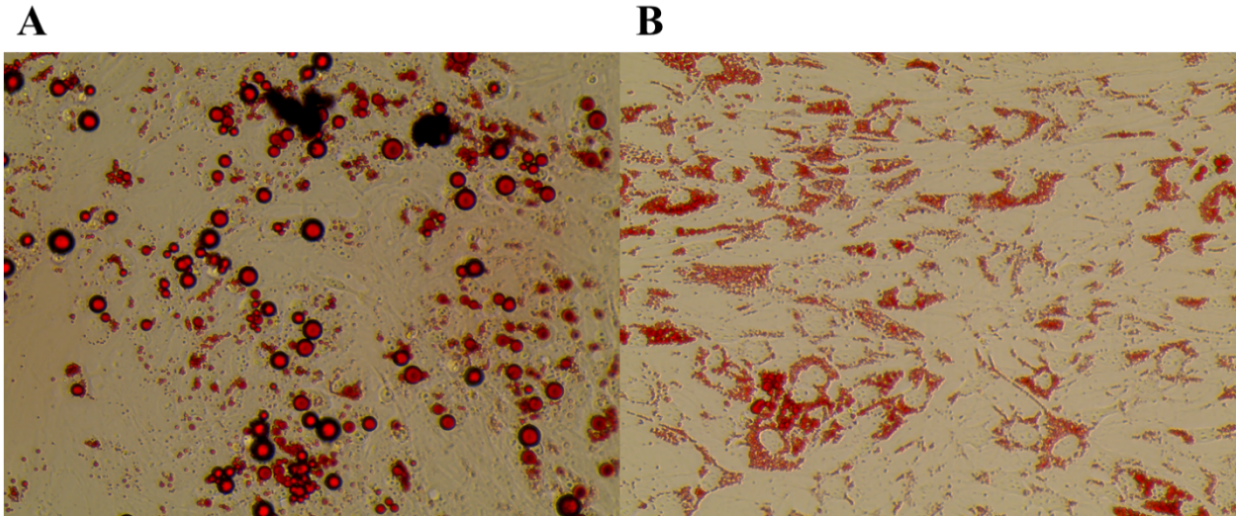

Figure 1. Microscopic morphologies of mature adipocytes derived from (A) dedifferentiated fat cells (DFAT) or (B) stromal vascular fraction (SVF) differentiated with 100 µM of c9-18:1 in 40x magnification.

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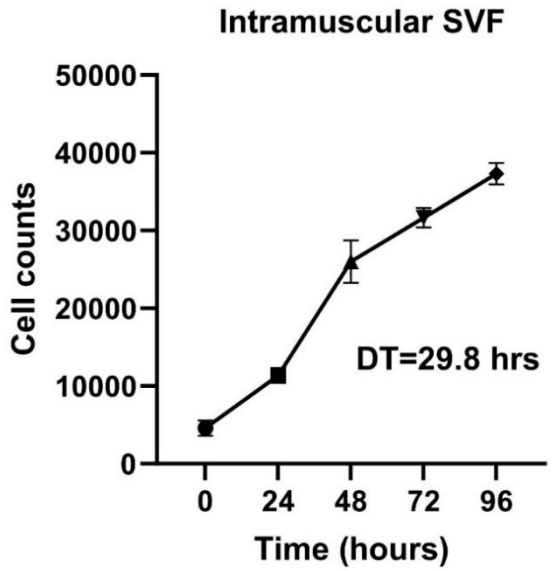

Figure 2. Growth curve of stromal vascular fractions (SVF) from 96 hours incubation measured by Cell Counting Kit-8 (Sigma Aldrich, Missouri, SL, USA). Stromal vascular fraction was seeded in density of 5000/wells. After 2 hours of incubation, cells were incubated with cell culture medium with 100 µl of water-soluble tetrazolium (WST) for 2 hours. Absorbance at 450 nm wavelength was measured by BioTek Synergy HT microplate reader (BioTek, Charlotte, VT,

USA) on daily basis to generate the growth curve. Doubling time was calculated from 0 to 96 hours duration.