EFFECTS OF METABOLIC MODIFIERS ON LIPOGENIC AND LIPOLYTIC GENE EXPRESSIONS IN SHEEP

By

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This thesis is dedicated to my late father,
Allahyarham Ab Aziz bin Mat, who went to meet his Creator on the day of his PhD registration. May Allah rest the departed soul in eternal peace of Jannah. This is the continuation of his dream.

ABSTRACT

The metabolic modifiers, which include growth hormone, beta-adrenergic agonists, and anabolic steroids, modify animal metabolism and affect the mechanism of nutrient absorption. This experiment aimed to investigate the effect of a beta-adrenergic agonist (BA) and growth hormone (GH) in sheep on processes involved in regulating lipid metabolism, in the tissues principally responsible for nutrient energy metabolism; adipose tissue (subcutaneous adipose tissue), skeletal muscles (*longissimus dorsi* (LD) and *supraspinatus* (SS)) and liver. In addition, the study sought to determine whether a transdifferentiated-myogenic cell culture system demonstrating myogenic and adipogenic characteristics would respond to these agents in a similar manner as *in vivo*.

Male lambs (120 days old) given *ad-libitum* feed were exposed for 6 days to bovine GH (bGH, 3.75mg/kg body weight, POSILAC, Monsanto) (n=10), the BA cimaterol (at 10mg/kg in the feed) (n=10) or a control C group (n=11). BA treatment significantly induced muscle hypertrophy, whilst GH increased liver weight without any significant effect on muscle weights. The increase in plasma IGF-1 in GH-treated lambs suggested that the GH effect was mediated by the IGF-1. There was no effect of both treatments on fatty acid profile in the SS muscle, whilst in subcutaneous adipose tissue, BA tended to increase the unsaturated fatty acids proportion ($P = 0.058$). The metabolomics analysis suggested the strong effect of GH on significantly elevating plasma NEFAs and glycerol concentrations ($P<0.05$). This was associated with significantly higher plasma concentration of the ketone body 3-hydroxybutyrate ($P<0.05$) and glucose ($P<0.001$). These changes were accompanied by reduced plasma glucogenic non-essential amino acid concentrations. Given the lack of increase in muscle mass, these have might contribute to the increase in plasma glucose level, in GH-treated lambs, in addition to the lipolytic effect of GH. This would suggest GH induced an insulin resistance. Similar effects were not observed in BA-treated sheep. In addition, in GH- and BA-treated lambs the reduction of muscle fatty acid synthase (FAS) mRNA ($P<0.01$) indicated a lowered lipogenic potential implicating the effect of both agents in developing leaner muscle.

Although, this effect was more pronounced in GH treated muscles with both acetyl CoA carboxylase (ACC) and FAS mRNA being reduced in subcutaneous adipose tissue.

The cell culture experiment was carried out using transdifferentiated-myogenic C2C12 cell lines. Expression of genes with associated with myogenesis and adipogenesis were induced, and fat droplets were observed. This suggested the presence of both myotubes and adipocytes in co-culture and thereby the establishment of a cell culture system analogous *in vivo* where the muscle has an intramuscular fat deposition. Upon the treatment with dibutyryl cyclic adenosine monophosphate (dbcAMP) and Des (1-3) insulin-like growth factor-1 (Des (1-3) IGF-1, there was a strong stimulatory effect of dbcAMP in regulating genes associated with myogenesis ($P<0.05$), which might contribute to the hypertrophic effect, whilst lipogenic gene expression was reduced ($P<0.05$). This indicated that this cell culture system exhibited the similar characteristics as had been observed *in vivo* in the sheep trial. Des (1-3) IGF-1 appeared to induce adipogenesis, while not affecting lipogenesis or lipolysis, suggesting different pathways of IGF-1 induction between *in vivo* or *in vitro*.

In conclusion, the BA strongly affects the muscle development and hypertrophy, whilst GH affects the lipid mobilisation and deposition. However, both agents were demonstrated to potentially affect metabolism which would impact of the production of lean muscle.

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ABBREVIATIONS

%	Percentage
µl	Microliter
3’	Three prime
5’	Five prime
AC	Adenylyl cyclase
ADP	Adenosine diphosphate
AMPK	Adenosine monophosphate kinase
ANOVA	Analysis of variance
AR	Adrenergic receptor
ATP	Adenosine triphosphate
BA	Beta-adrenergic agonist
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
cm	Centimetre
Cp	Crossing point
CSA	Cross-sectional area
dbcAMP	Dibutyryl-cyclic adenosine monophosphate
DEX	Dexamethasone
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNL	*De novo* lipogenesis
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FAME	Fatty acid methyl ester
FBS	Fetal bovine serum
g	Gram
g/kg	Gram per kilogram
GC/MS	Gas chromatography/mass spectrometry
gDNA	Genomic deoxyribonucleic acid
GH	Growth hormone
IBMX	Isobutylmethylxanthine
IGF-I	Insulin-like growth factor 1
IGFs	Insulin-like growth factors
IMF	Intramuscular fat
LCFA	Long chain fatty acid
LC/MS	Liquid chromatography/mass spectrometry
LD	*Longissimus dorsi*
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeters
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acid

NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NEFAs	Non-esterified fatty acids
ng	Nanogram
nm	Nanometers
^{o}C	Degrees Celsius
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
ppm	Parts per million
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Rotation per minute
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SDS	Sodium dodecyl sulphate
sec	Second
SED	Standard error of difference
SEM	Standard error of mean
SFA	Saturated fatty acid
SS	*Supraspinatus*
ST	*Semitendinosus*
TLC	Thin layer chromatography
TAE	Tris-acetate-EDTA
U	Units
VIN	*Vastus intermedius*
VLAT	*Vastus lateralis*
v/v	Volume per volume
w/v	Weight per volume

1. INTRODUCTION

The world's population has been projected to increase by 30 percent and may reach 9.6 billion by 2050 (FAO, 2009a). This situation will lead to an increase by up to 70 percent in food demand, along with other factors, such as rising income and urbanisation, which contribute to the increase consumption of food per capita and dietary changes towards increased desire for high-quality animal protein such as milk and meat (Dunshea et al., 2016; Valin et al., 2014). This demand will challenge the agricultural industries and animal scientists to enhance productivity, by producing more milk and increasing average daily gain for meat animals, but in a more efficient way. One of the crucial targets is to increase animal nutrient utilisation efficiency, by reducing the quantity of feed per unit output (CAST, 2005; National Research Council, 1994). To achieve this, metabolic modifiers, such as growth hormone (GH) and beta-agonist (BA), have been developed to increase feed efficiency by improving performance and lean content in the animals, and thereby increasing profitability (Dikeman, 2007; Ricks et al., 1984).

Generally, the mechanism of action of these metabolic modifiers is to increase protein accretion and reduce fat deposition (Dunshea et al., 2005). They are also referred to as repartitioning agents, as they redirect nutrients away from adipose tissue and gastrointestinal tract towards mammary glands for milk production and skeletal muscle for muscle accretion (Ricks et al., 1984). In terms of lipid metabolism, BA may have a direct effect by increased lipolysis and reduced lipogenesis but also increase protein deposition (de Almeida et al., 2012; Dunshea et al., 2005; Mersmann, 2002). Similar effects are observed for GH, as it also improved protein deposition, and reduced fat accretion but also improved milk synthesis efficiency (Dunshea et al., 2016). Thus, this may explain their role in modifying composition of fat, and affecting the toughness and eating quality of the meat (Ladeira et al., 2016; Dikeman, 2007; Warriss et al., 1991). As the fat content in meat is considered unhealthy, the usage of the metabolic modifiers could be beneficial for the consumer, by producing healthier products (CAST, 2005). However, improvement of producing lean meat by these

metabolic modifiers have been demonstrated to negatively impact on meat quality, probably through the reduction of intramuscular fat (IMF) (Dunshea et al., 2005).

Although the metabolic modifiers have been demonstrated to improve performance and carcass characteristics, the mechanism in the molecular level by which these agents control lipid metabolism remain poorly defined. Thus, in this study, we hypothesized the mechanism in controlling lipid metabolism by BA and GH, was reflected by the transcription of the key lipogenic and lipolytic genes, which associated with reduced fat deposition, both *in vivo* and *in vitro*. The overall aim of this project was to investigate the mode of action of these metabolic modifiers (BA and GH) in affecting sheep lipid metabolism, particularly on lipogenesis and lipolysis. In this study, the molecular approach was used to determine the relationship between the skeletal muscle (*suprasupinatus*, (SS) and *longissimus dorsi*, (LD)), liver, and subcutaneous adipose tissue, in respect to their role in controlling lipid metabolism, as they are involved in both nutrient partitioning and deposition (Frayn et al., 2006). In addition, we also utilized the transdifferentiated-myogenic cells, into 'adipo-like' cells, as a muscle model, to elucidate the effects of both agents in altering IMF and myogenesis. The response to BA and GH of key metabolic genes were identified, and possible mechanisms were evaluated in order to improve understanding of fat deposition in skeletal muscle tissue.

2. LITERATURE REVIEW

2.1 FOOD SECURITY AND GLOBAL FOOD DEMAND

2.1.1 World population and increase food demand

Increasing world population and the associated rise in per capita income, especially from the low-income countries, will result in a change in food consumption from lower value cereals (fewer grains) to higher value livestock products (rising consumption of meat, milk and eggs) (Dunshea et al., 2016; Regmi et al., 2001). Since the early 1980s, total meat and milk consumption has grown at 6 and 4% per year, respectively, throughout the developing countries. For example, in East and Southeast Asia — income grew at 4 - 8%, and meat consumption grew between 4 - 8% per year (Delgado, 2003). Urbanization of the population increases the demand of agriculture products supply to urban populations (Satterthwaite et al., 2010). Urbanization has considerable impact on patterns of food consumption in general, and there is plenty of evidence that increases in livestock product consumption may be associated with increases in urbanization rates, which is also associated with an improvement of trading infrastructure, which allows these perishable goods to be traded more widely (Thornton, 2010).

In most communities, meat has long occupied a special place in the diet, for a variety of reasons including taste preference, prestige, tradition and availability, with the nutritional aspects being included more recently. While it is true that meat is not essential in the diet, and many people thrive on diets derived largely or even entirely from plant foods (so long as the amounts and variety are sufficient), there are many diets that would be considerably improved by the inclusion of even small amounts of meat and meat products. This is because, compared with plant foods, they contain an essential amino acid profile which closely matches that requirements of humans and a range of associated vitamins and mineral salts. As little as 25 g of meat will supply nearly half of a child's daily need for protein and half the vitamin B12; the addition of 100 g of meat to

the average Zambian diet would increase the protein by half, iron by 12%, niacin by 40% and energy by 25 % (FAO, 2013). Apart from supplying additional protein the amino acids in that protein complement the cereal sources of protein by making good their relative deficiency of lysine. Moreover, compared with plant foods, the iron in meat is well absorbed and meat promotes the absorption of iron from other foods (FAO, 2013).

2.1.2 Production efficiency

Efficiency is the ability in producing desired yield with the least amount of input (time, effort, expenses) (Jensen, 2007). As far as the agricultural productivity is concerned, there are several techniques to increase the production efficiency. In terms of livestock production, cereals are the major constituents in the animal feeds and the availability of this commodity directly affects the production and consumption of the livestock products. The upsurge in meat consumption is reflected in the increase in cereal production and global cereal demands, if this demand is not met there is the potential for a global cereal shortage (Nonhebel and Kastner, 2011; Keyzer et al., 2005). Higher grain prices inevitably lead to the higher feed cost. Currently feed costs represent approximately 70% of animal production costs this is partially due to there being low efficiency in food conversion by the food-producing animals (CAST, 2005).

Therefore, animal scientists must develop new technologies to increase yields and production efficiency (i.e., yield of milk/feed input; yield of muscle/feed input), produce leaner animals, thereby enabling increased economic return on investment to the producer (Lawrence et al., 2008; CAST, 2005). The development and use of metabolic modifiers to enhance the meat and milk production might be part of the strategy that could achieve this (Dunshea et al., 2016; FAO, 2013), which have proved efficiency.

2.2 MUSCLE AND ADIPOSE DEVELOPMENT AND GROWTH

2.2.1 The relationship between muscles and meat

Commonly meat is defined as those animal tissues (flesh and organs) that are suitable for use as food. The flesh of cattle, pigs and sheep is distinguished from that of poultry by the term red meat, while the flesh of poultry (such as chicken, turkey, duck, pigeon, and guinea fowl) is termed white meat (FAO, 2013). This includes all processed and manufactured products that might be prepared from these tissues. Generally, the composition of meat, after rigor mortis but before post-mortem degradative changes, can be approximated to 75% water, 19% protein (myofibrillar proteins, sarcoplasmic proteins that consists of myoglobin, and stroma proteins that consists of collagen and elastin), 3.5% soluble, non-protein substances (amino acids, vitamins, carbohydrate (organic), and minerals such as phosphorus, potassium, and small amount of sodium, magnesium, calcium and zinc) and 2.5% fat (Frontera and Ochala, 2014; Olaoye, 2011). Thus, meat is a good source of protein as well as essential amino acids, minerals and vitamins. The variation in this composition plays an important role in determining meat quality.

Meat quality is in part determined by its compositional quality (lean to fat ratio), which includes the amount of marbling, as well as the palatability factors such as visual appearance, smell, firmness, juiciness, tenderness and flavour (Bender, 1992; Marchello and Dryden, 1968). The mechanism controlling meat quality development often associated with altered post-mortem muscle metabolism, which involved in the changes of glycolysis rate, which can create unfavourable muscle pH, and subsequently will result in protein denaturation and affect the quality parameters (Ryu and Kim, 2005).

The myofibrillar protein constitutes about 11.5% of total protein in meat. Some studies have reported that the size and number of muscle fibres are factors that affect meat quality as the increasing size and number of fibres was negatively correlated to drip loss and tenderness (Lee et al., 2010; Choi and Kim, 2009). In pigs, higher total number of fibres within a muscle tended to exhibit smaller fibre

sizes, and exhibited higher muscle pH45 min (pH measured at 45 min post-mortem) and lower drip loss (fluid lost from fresh, non-cooked meat via passive exudation) than pigs with a lower total fibre number and large fibre size. Moreover, the total amount of type I fibre is negatively correlated with drip loss and lightness (Choi and Kim, 2009). They also suggested the positive correlation between fibre size and intramuscular fat content. The intramuscular fat (located within the perimysium which surrounds muscle fibres) also is a major factor in meat quality and associated with tenderness, juiciness and palatability, it also contains flavour-forming precursor material and positively correlated with meat quality and taste (Jeong et al., 2012; Liu, et al., 2012; McPhee et al. 2008). Fatty acids, especially polyunsaturated fatty acids (PUFA), are major flavour precursors of meat (Yu et al., 2013).

In agriculture, the meat animals are raised for their muscle (Du et al., 2015). From the structure and the development of meat described above, we summarized that there is a direct connection between meat production and the skeletal muscle development, which primarily involved myogenesis, while another process called adipogenesis that also plays a major part particularly in the intramuscular fat development, which will be discussed briefly below, and will be focused more on the molecular level. Another process in the lipid metabolism of intramuscular fat deposition, such as lipogenesis and lipolysis will also be discussed to give an idea of the whole process in muscle, which might give an impact in the skeletal muscle development, and quality of meat.

2.2.2 Myogenesis

Myogenesis (formation of muscle), can be defined as a developmental process of myofibres, the structural units for skeletal muscle. This involves the commitment and differentiation of mesenchymal stem cells, their conversion into muscle cells, and their fusion for the formation of the multinucleated fibres or myotubes. Myogenesis begins prenatal. During the early embryonic stage, primary fibres arise, and this is followed by the formation of secondary fibres during fetal stage (Yan et al., 2013).

As the mesenchymal stem cells can be committed into either myogenic, adipogenic or chondrogenic lineages, specific protein regulators are responsible in the process of determination, including the wingless (Wnt) protein family, the hedgehog (Hh) protein family, paired box transcription factors Pax3 and Pax7, and bone morphogenic factors (BMPs) (Yan et al., 2013; Bentzinger et al., 2012). As described by Ladeira et al. (2016), during the determination stage the mesenchymal stem cells give rise to myogenic regulatory factor five expressing-myogenic factor 5 (Myf5(+)) and non-expressing cells (Myf5(-)). The Myf5(+) will develop to muscle cells, and Myf5(-) to white adipoblasts. Their further development will be controlled by the regulators as stated above.

Wingless/int (Wnt) family protein is an extracellular signalling molecule, which act through the Fridzzled (Fzd) receptors, 'canonically' transduced the β-catenin-dependent mechanism, and form a complex with nuclear T-cell specific transcription factor (TCF). This can then induce target gene transcription. However, this can also occur via a non-canonical, β-catenin-independent mechanism (van Amerongen and Nusse, 2009). Wnt plays an important role in activating myogenic differentiation (Jin et al., 2016; Ladeira et al., 2016; Cisternas et al., 2014; Bertzinger et al., 2012; Han et al., 2011; Cossu and Borello, 1999). Han et al. (2011) demonstrated the activation of Wnt signalling, enhanced mRNA expression and activity of Myf5 *in vitro*.

The hedgehog (Hh) protein, particularly the Sonic hedgehog (Shh), instead of Indian hedgehog (Ihh) and Desert hedgehog (Dhh), is also a coactivator in myogenesis, including stimulation of myogenic regulatory factors (MRF) expression, such as Myf5 (Gustafsson et al., 2002; Borycki et al., 1999). The interaction of Hh with the Patched receptor will stimulate the release of the smoothened molecule and following nuclear translocation through GLI transcriptional factors (GLI1 and GLI3) will eventually be responsible for the transcription of the target genes, such as upregulation of Myf5 and MyoD expression (Jin et al., 2016; Bertzinger et al., 2012; Lum and Beachy, 2004; Borycki et al., 2000).

The paired box transcription factors Pax3 and Pax7 are involved in developmental and adult regenerative myogenesis. They are known as early markers of the developing dermomyotome, which is the source of future myoblasts. In the Pax3:Pax7-double knockout, there is a major defective in skeletal muscle formation (Relaix et al., 2005). The expression of Pax3 and Pax7 in mesenchymal stem cells induces the expression of Myogenic Regulatory Factors (MRFs), which leads to the myogenic differentiation.

In contrast, the bone morphogenic protein (BMPs) is a negative regulator in myogenesis, which inhibits the expression of myogenic genes. BMPs are the growth factors that belong to the subclass of the transforming growth factor β (TGFβ) superfamily. BMPs play an important role in the commitment of mesenchymal stem cells (MSCs) into cell lineages, such as adipogenic, and osteogenic lineage (Ladeira et al., 2016; Beederman et al., 2013), and early bone formation (Tsumaki and Yoshikawa, 2005).

2.2.2.1 Myogenic Regulatory Factors (MRFs)

The four Myogenic Regulatory Factors (MRFs) are often categorized as master regulators of myogenic determination and differentiation, and consist of highly conserved basic DNA-binding domain and helix-loop-helix transcription factors, which are required for the sequence-specific DNA binding and heterodimerization with E proteins to the E-box sequence (CANNTG), for the activation of gene transcription (Sabourin and Rudnicki, 2000). They include the myogenic factor 5 (Myf5), myogenic differentiation 1 (MyoD), myogenic factor 6 (MRF4), and myogenin (MyoG) (Endo, 2015). The studies involving the null mutations of these MRFs has demonstrated their effect in the muscle formation. In the MyoD-knockout mice, there was a normal development of muscle phenotype, along with the increase of Myf5 expression (Rudnicki et al., 1992). However, there was a perinatal death in the Myf5-knockout mice, with abnormal rib development (Braun et al., 1992). The MyoD/Myf5-double knockout mice fails to develop any skeletal muscles, and die at birth (Rudnicki et al., 1993). In the effect of MRF4 deficiency, the mice showed normal development of muscle, along with increase in myogenin expression (Braun et al., 1995; Patapoutian et

al., 1995; Zhang et al., 1995). However, after the inactivation of myogenin, the mice have normal myoblasts, but fail to develop into myofibres, and die at birth, therefore indicating that myogenin is a differentiation factor rather than a muscle development factor (Eng et al., 2013; Hasty et al., 1993; Nabeshima et al., 1993). Thus, from these findings, Myf5 and MyoD are classed as primary MRFs, which are expressed earlier during determination step of the precursor cells to the myogenic lineage, thereby converting into myoblasts. Myogenin and MRF4, are considered to be secondary MRFs, involving in the terminal differentiation of myoblast differentiation into myocytes and myotubes formation (Yan et al., 2013; Sabourin and Rudnicki, 2000). The developmental process of myogenesis involving MRFs is shown in Figure 2.1.

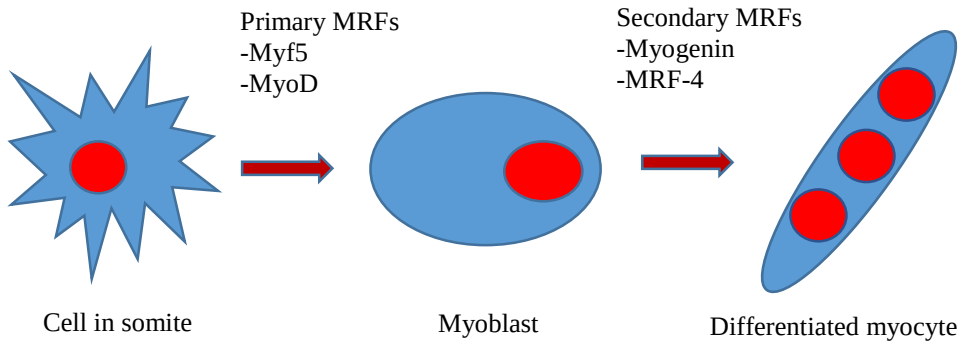


Figure 2.1: Primary MRFs: MyoD and Myf-5, involved during commitment step of the proliferating somitic cells (progenitor cells) to the myoblasts. The secondary MRFs: myogenin and MRF4 induced the proliferation and differentiation of myoblasts into myocytes, and fusion into myofibers (Adapted from Sabourin and Rudnicki, 2000).

2.2.3 Adipogenesis

Adipogenesis is a two-step developmental processes in which pluripotent mesenchymal stem cells (MSCs) differentiated into preadipocytes, undergo a secondary differentiation of preadipocytes into mature, insulin-responsive adipocytes (Ali et al., 2013). During the initial commitment stage, in regard to adipogenesis, the MSC give rise to the non-expressing myogenic factor (Myf5(-)) cells, which will develop into white adipoblasts, instead of expressing myogenic factor 5 (Myf5(+)) cells, which will develop into muscle and brown

adipocytes (Ladeira et al., 2016). Further development of these cells to myogenic or adipogenic were controlled by several different regulators such as activating protein-1 (AP-1), single transducers and activators of transcriptors (STATs), sterol regulatory element-binding protein-1(SREBP-1), zinc finger protein (ZFP) family, wingless and INT-1 protein (Wnts) family, Kruppel-like factors (KLFs), bone morphogenic protein (BMP) family, fibrolast growth factors (FGFs) family and preadipocyte factor 1 (Pref-1) (Ladeira et al., 2016; Sarjeant and Stephens, 2012; Siersbæk et al., 2011).

During the next stage of adipogenesis, the preadipocytes differentiate into adipocytes, which is controlled by two key regulators, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs) specifically C/EBPα. Adipose tissue is the primary sites for the PPARγ expression, and it induced early differentiation of adipocytes, while C/EBPα, which is expressed in many tissues, induces terminal adipocyte differentiation. When co-expressed, both factors act synergistically to powerfully activate developmental process of adipocytes in fibroblastic cells (Hu et al., 1995). As with skeletal muscle, adipocyte differentiation requires the expression of genes which carry out roles fundamental to the function of adipocyte and are associated with the adipogenesis program, these include fatty acids binding protein 4 (FABP4), adiponectin, and fatty acid synthase (FAS), which induced the mature adipocyte formation (Moseti, et al., 2016).

2.2.3.1 PPARs

Peroxisome proliferator-activated receptors (PPARs) are a nuclear hormone receptor superfamily. They are characterized as an "orphan" receptors which belong to the nuclear receptor group which includes liver X receptor (LXR), These receptors are activated by the lipophilic ligands, such as the derivatives of long-chain polyunsaturated fat which includes prostaglandins, and synthetic arachidonate leukotrienes (such as 5,8,11,14-eicosatetraynoic acid (ETYA)), as well as anti-diabetic drugs such as thiazolidinediones (TZD) including troglitazone, which then promote peroxisome proliferation (Moseti et

al., 2016; Siersbæk et al., 2010; Farmer, 2005; Hu et al., 1995). PPARs family is composed of three different members (PPARα, β/δ, γ). PPARα is predominantly expressed in the liver, heart, kidney, and bone, and involved in the nutrient metabolism such as gluconeogenesis, and amino acid and lipid metabolism, especially in the beta-oxidation of fatty acids (Tyagi et al., 2011; Brun et al., 1996). PPARδ are present ubiquitously expressed in many tissues, and have been suggested to regulate energy expenditure and pre-adipocyte differentiation, whilst PPARγ is mostly expressed in adipose tissues, and involved in energy storage, and facilitate early adipogenesis (Moseti et al., 2016; Tyagi et al., 2011, Hu et al., 1995).

Studies by Brun et al. (1996) also showed that only PPARγ is the predominant factors when combined with C/EBPα, to promote adipogenesis. PPARα is, at slower rate than PPARγ, capable of stimulating significant adipocyte differentiation. However, PPARδ does not stimulate adipocyte differentiation possibly because it has the weakest relative binding to with gDNA regulatory element sequences (DR-1 type elements) of fat specific genes.

The treatment of clonal preadipocytes, 3T3-F442A cells from mouse, with PPAR agonist, pioglitazone, resulted in increased lipid accumulation and higher triglyceride content in the mature adipocytes (Sandouk et al., 1993). In humans, the role of PPARγ in regulating adipogenesis, is demonstrated by its ability to stimulate fatty acid binding protein-4 (FABP4) expression (which will be discussed next), a key protein required for adipocyte function (Qian et al., 2010).

2.2.3.2 C/EBPs

Along with PPARs, the CCAAT/enhancer-binding proteins (C/EBPs) also induce the adipogenic program, by promoting terminal differentiation and maturation of adipocytes (Hu et al., 1995). There are six members of C/EBPs (α, β, δ, γ, ε, and ζ), which belongs to a class of highly conserved basic-leucine zipper (bZIP) DNA-binding proteins for dimerization with other C/EBPs and other transcription factors (Sarjeant and Stephens, 2012). Three members, (α, β,

δ) have been shown to induce adipocyte differentiation. C/EBPβ and C/EBPδ are regulated in early stage of adipogenesis, and induced by hormonal stimulants 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone, respectively, which are the potent stimulators for terminal differentiation (Sarjeant and Stephens, 2012; Yeh et al., 1995; Cao et al., 1991). Both can cross-dimerize with one another and also transactivate C/EBPα (Sarjeant and Stephens, 2012; Cao et al., 1991). *In vitro* experiments using 3T3-L1 cells confirmed that inhibition of these C/EBPs blocked the terminal differentiation and attenuated adipogenesis (Yeh et al., 1995). Meanwhile, *in vivo* studies using mice lacking either C/EBPβ and/or C/EBPδ resulted in defective development of adipose tissue (Tanaka et al., 1997) and C/EBPα-deficient mice reduced the number lipid droplets compared with control mice (Wang et al., 1995).

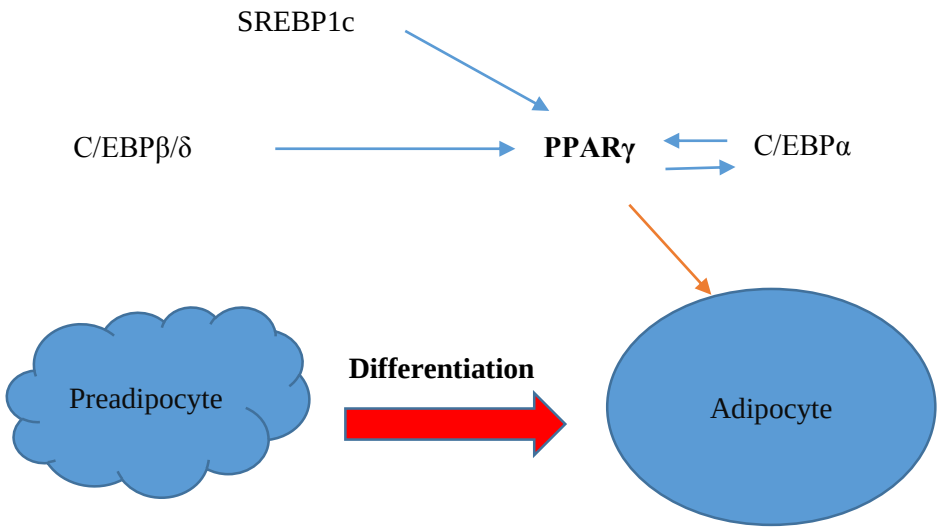


Figure 2.2: The role of PPARγ in adipogenesis. Ligand stimulated-PPARγ modulates and stimulates C/EBPα expression, which in turn cooperate to allow a positive feedback which maintains PPARγ expression, as well as inducing other adipogenic genes. Both PPARγ and C/EBPα are involved in adipogenesis, including stimulating adipocytes genes and insulin sensitivity. Activation of PPARγ induces terminal differentiation by stimulating other target genes (Adapted from Tortonoz and Spiegelman, 2008).

2.2.3.3 FABPs

The mammalian fatty acid binding proteins (FABPs) belong to the ubiquitous, multigene intracellular or cytoplasmic lipid-binding protein family, which bind hydrophobic ligands such as long-chain fatty acids, eicosanoids, leukotrienes, and prostaglandins, with high affinity and mobilize them throughout the cells (Smathers and Petersen, 2011; Storch and Thumser, 2010; Elmasri et al., 2009). The family includes nine different FABPs, named after the tissue-specific distribution, and have been identified as: liver-(L-FABP; FABP1), intestinal-(I-FABP; FABP2), heart/muscle-(H-FABP; FABP3), adipocyte-(A-FABP; FABP4), epidermal-(E-FABP; FABP5), ileal-(Il-FABP; FABP6), brain-(B-FABP; FABP7), myelin-(M-FABP; FABP8) and testis-(T-FABP; FABP9) (Smathers and Petersen, 2011; Storch and Thumser, 2010, Haunerland and Spener, 2004; Hertzel and Bernlohr, 2000) and newly discovered FABP12 (Liu et al., 2008). FABP tissue distributions were shown in Table 2.1.

Table 2.1: FABP Tissue Distribution (Adapted from Smathers and Petersen, 2011)

Gene	Common name	Localisation
FABP1	Liver FABP	Liver, intestine, pancreas, kidney, lung, stomach
FABP2	Intestinal FABP	Intestine, liver
FABP3	Heart FABP	Cardiac and skeletal muscle, brain, kidney, lung, stomach, testis, adrenal gland, mammary gland, placenta, ovary, brown adipose tissue
FABP4	Adipocyte FABP	Adipocytes, macrophages, dendritic cells, skeletal muscle fibres
FABP5	Epidermal FABP	Skin, tongue, adipocyte, macrophage, dendritic cells, mammary gland, brain,

		stomach, intestine, kidney, liver, lung, heart, skeletal muscle, testis, retina, lens, spleen, placenta
FABP6	Ileal FABP	Ileum, ovary, adrenal gland, stomach
FABP7	Brain FABP	Brain, central nervous system (CNS), glial cell, retina, mammary gland
FABP8	Myelin FABP	Peripheral nervous system, Schwann cells
FABP9	Testis FABP	Testis, salivary gland, mammary gland
FABP12	-	Retinoblastoma cell (H), retina (ganglion and inner nuclear layer cells) (R), testicular germ cells (R), cerebral cortex (R), kidney (R), epididymis (R)

*(H) = human, (R) = rat

However, FABPs are not only specific for certain tissues, but are also expressed in various locations (Smathers and Petersen, 2011; Haunerland and Spener. 2004). The FABPs are involved in facilitating fatty acid influx across cell membrane (ingestion) and the movement of fatty acids in the cells to the specific cellular compartment and organelles. They are also involved in lipid signal transduction and solubilization, for the modulation of enzyme reaction and regulation of gene expression, proliferation and differentiation of the cells (Gan et al., 2015; Storch and Thumser, 2010; Elmasri et al., 2009; Chmurzyñska, 2006; Haunerland and Spener, 2004; Zimmerman and Veerkamp, 2002; Hertzel and Bernlohr, 2000).

Adipocyte fatty acid-binding protein (AFABP), also known as FABP4 as well as aP2, as it has high sequence similarity with the myelin P2 protein (Furuhashi

et al., 2014). FABP4 is expressed primarily in adipose tissue, most abundantly in mature adipocytes and macrophages (Queipo-Ortuño et al., 2012; Maeda et al., 2005; Fu et al., 2000; Coe and Bernlohr, 1998) and thus, the FABP4 might act as an important mediator between these two components in adipose tissue (Queipo-Ortuño et al., 2012). The FABP4 expression is highly induced during adipocyte differentiation and during the transdifferentiation of myoblasts to adipocytes by the treatment with fatty acids (Taylor-Jones et al., 2002; Grimaldi et al., 1997), however can be affected by long-chain fatty acids (LCFA), oxidized low-density lipoprotein (OX-LDL), PPARγ and insulin (Gan et al., 2015). Therefore, the induction or increased expression of FABP4 is a good marker gene of cells increasing their "adipogenic" activity.

2.2.4 Lipogenesis

Fat accumulation is determined by the balance between lipogenesis (fat synthesis) and lipolysis (fat breakdown/fatty acid oxidation). *De novo* lipogenesis (DNL) is defined as an enzymatic process for the conversion of excess dietary carbohydrate into fatty acids. These can then be incorporated into triglycerides for energy storage, phospholipid synthesis for the structural component of the cell membrane, or can act as a signalling molecule (Ameer et al., 2014; Colbert et al., 2010; Hellerstein, 1999). DNL primarily occurs in adipose tissue in ruminant, liver in human, and both liver and adipose tissue in rodents and rabbit (Laliotis et al., 2010; Kersten, 2001; Ingle et al., 1972). In contrast, DNL in the chicken and pigeon occurs predominantly in the liver with adipose tissue acting merely as a depot site for preformed fats. In DNL, the synthesis of fatty acids occurs from various precursors, mainly from glucose and amino acids which produce acetyl-CoA during their catabolism. Therefore, these metabolites are susceptible to be converted to fatty acids in the intermediary metabolism (Schutz, 2004). The fatty acids are synthesized by an extra-mitochondrial system, which is responsible for the complete synthesis of palmitate from acetyl-CoA in the cytosol. They are then used as a substrate for further fatty acid synthesis through elongation and/or desaturation which takes place in the endoplasmic reticulum via the interaction of many catalytic enzymes (Laliotis et al., 2010).

DNL is regulated by two master regulators; sterol regulatory element binding protein-1 (SREBP-1) and carbohydrate responsive element binding protein (ChREBP) which are induced in response to insulin and glucose respectively (Postic and Girard, 2008). These regulators will activate the transcription of key lipogenic genes. The two key lipogenic genes are; acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which play a role in the transfer of acetyl-CoA from the mitochondrion to the cytosol (Towle et al., 1997). ACC is a rate-limiting and biotin containing cytoplasmic enzyme that catalyses the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, whereas the FAS catalyses the conversion of acetyl-CoA and malonyl-CoA to palmitic acid in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) (source of reducing equivalents) (Laliotis et al., 2010). The simplified diagram for DNL pathway is shown in Figure 2.3.

2.2.4.1 ACC

Acetyl-CoA carboxylase (ACC) mediated the rate-limiting and first committed step in DNL, catalysing the acetyl-CoA carboxylation, with ATP-dependent, to form malonyl-CoA, which is an important substrate for subsequent reaction by fatty acid synthase (FAS) for synthesis of long-chain fatty acids (Brownsey et al., 2006).

ACC is encoded by two major isoenzymes designated ACC-α (or ACC-1, gene) and ACC-β (or ACC-2, gene) in mammals. ACC-1 is a cytosolic isoform that is thought to be the predominantly controlling fatty acid synthesis, whereas ACC-2 is mitochondrial and involved in localizing malonyl-CoA production and which can interrupt carnitine palmitoyltransferase (CPT)-1 function, thus preventing the mitochondrial β-oxidation (Svensson et al., 2016; Postic and Girard, 2008). Despite expressed in all tissues, ACC-1 expression is highly inducible in tissues with highly lipogenic activity, such as liver, adipose tissue and lactating mammary gland. In contrast, ACC-2 is predominantly expressed in tissues that utilize fatty acid as an energy source, such as skeletal muscle and

heart (Laliotis et al., 2010; Postic and Girard, 2008). Thus, it is believed that only ACC-1 is committed in DNL.

2.2.4.2 FAS

Fatty acid synthase (FAS) is the central multi-domain enzyme complex in DNL, catalysing the condensation of acetyl-CoA (as a primer) and malonyl-CoA (as a carbon donor), initiated by the covalent binding with the enzyme, for the synthesis of palmitate, the 16-carbon saturated fatty acid, in the presence of NADPH (Hogan and Stephens, 2005; Chirala et al., 2001; Katiyar et al., 1975). However, another products also generated from the reaction such as stearate, myristate, laureate, and even shorter-chain fatty acid (Ameer et al., 2014; Zhu et al., 2014). The fatty acids then undergo modification by subsequent elongation and/or desaturation reaction for the formation of complex lipids for different functions such as formation of cell membrane, storage of energy, and formation of signalling molecules (Svensson et al., 2016).

The FAS expression is controlled primarily by the transcriptional induction, through the effects of nutritional and hormonal signals. FAS mRNA levels are frequently used as markers of DNL. Study by Claycombe et al. (1998) using human adipocytes demonstrated the increase by five-fold in FAS mRNA and induced FAS gene transcription and expression with the treatment of insulin.

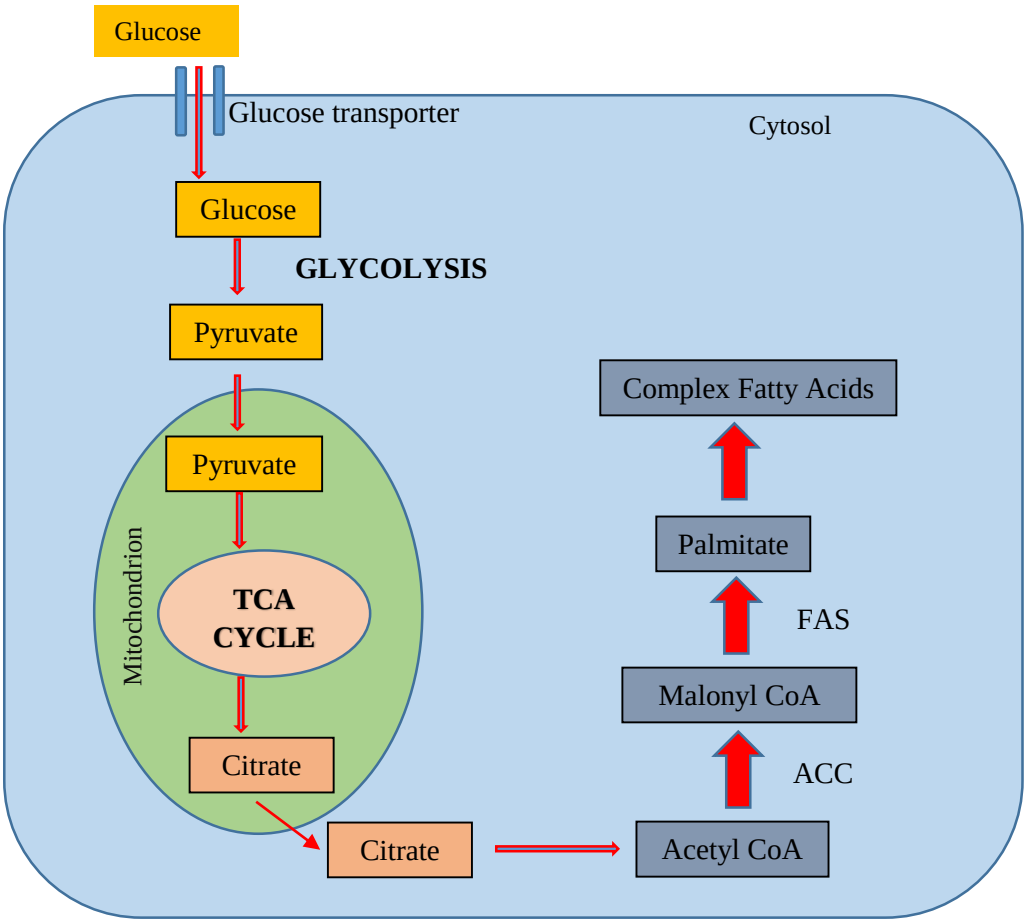


Figure 2.3: The process of *de novo* lipogenesis (DNL): The glucose transporter provides the glucose for the glycolysis and subsequent pyruvate formation, which is transformed into acetyl-CoA, and available for TCA cycle. The ATP-citrate lyase (ACLY) catalyse the conversion of citrate (from TCA) intracellularly into acetyl-CoA, which then take part in the fatty acid synthesis catalyse by Acetyl-CoA carboxylase (ACC), for the production of malonyl-CoA, and then Fatty acid synthase (FAS) generates for the palmitate, which is subsequently can be used for complex fatty acid synthesis by elongate, and/or desaturation (Adapted from Ameer et al., 2014).

2.2.5 Lipolysis

Lipolysis is defined as the catabolism or hydrolysis of triacylglycerols (TGs) stored in cellular lipid droplets, to generate non-esterified fatty acids (NEFAs) and glycerol (Zechner et al., 2012; Duncan et al., 2007). According to Lass et al. (2011), specifically, lipolysis is the process of the hydrolysis of primary and secondary ester bonds between long-chain fatty acids and the glycerol backbone. It occurs in essentially all tissues and cell types, but it is most abundant in white and brown adipose tissue. White adipose tissue is the major energy reserve in

mammals. The full hydrolysis of TGs depends on the activity of three enzymes; adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL). ATGL belongs to the family patatin domain-containing proteins, which preferentially catalyse the initial step of lipolysis, converting TGs to diacylglycerols (DGs) and NEFAs, HSL is rate-limiting for DGs catabolism. It is a multifunctional enzyme capable of hydrolysing a variety of acylesters including TGs, DGs, and momonoacylglycerols (MGs) and MGL hydrolyzes MGs (Zechner et al., 2012). The pathway for lipolysis is shown in Figure 2.4

2.2.5.1 ATGL

Adipose Triglyceride Lipase (ATGL), also known as patatin-like phospholipase domain containing protein-2 (PNPLA-2) is selectively responsible for the initial and rate-limiting step in catalyses the hydrolysis triglyceride (TAG) molecules producing diglyceride (DAG), subsequently a substrate for hormone sensitive lipase (HSL), and free fatty acid (Zimmermann et al., 2004). The expression of murine ATGL mRNA and enzyme activity are highest in white and brown adipose tissue, and also observed in testis, cardiac muscle, skeletal muscle (predominantly in type I fibres), and liver, as well as high levels of human ATGL were found in adipose tissue (Zechner et al., 2009; Zimmermann et al., 2009; Jocken et al., 2008; Reid et al., 2008; Zimmermann et al., 2004). The ATGL expression is markedly upregulated during murine 3T3-L1 adipocyte differentiation (Kershaw et al., 2006; Kim et al., 2006). Haemmerle et al. (2006) reported the ATGL-knockout mice, exhibited the reduced fatty acid released from adipose tissue and reduced fatty acid plasma levels, suggesting reduced rate of lipolysis. They also observed high triglyceride accumulation in non-adipose tissues including the testis, skeletal and cardiac muscle. In human, the ATGL mutations in the ATGL gene are associated with systemic triacylglycerol accumulation and cardiac abnormalities, as well as hepatomegaly, this inherited condition is known as "*Neutral Lipid Storage Disease with myopathy* (NLSDM)" (Lass et al., 2011).

The full activation of ATGL activity requires interaction with its coactivator protein annotated as α/β hydrolase fold domain-containing protein-5 (ABHD5), also known as comparative gene identification-58 (CGI-58) (Lass et al., 2006), while its activity is decreased by a peptide called G0/G1 switch gene 2, isolated from mononuclear cells (Yang et al., 2010b).

2.2.5.2 HSL

Earlier in 1960s, a lipase in white adipose tissue (WAT) was discovered which was thought to be stimulated by hormones and therefore given the name hormone sensitive lipase (HSL). The hormone sensitive lipase has previous been considered as the only enzyme that involved in triglyceride hydrolysis (Vaughan, et al., 1964; Hollenberg et al., 1961). The review by Lass et al. (2011) indicated the hydrolysis rates by HSL of DAG is ten times fold faster than TAG and monoglyceride (MAG), suggesting that HSL is predominantly a DAG hydrolase rather than TAG hydrolase. This is supported by the findings in HSL-deficient mice, which showed no accumulation of TAG, instead of large accumulation of DAG in many tissues (Haemmerle et al., 2002; Osuga et al., 2000). Currently, HSL been accepted as a rate-limiting enzyme capable for hydrolyse DAG, TAG, MAG, cholestryl esters, retinyl esters, and short-chain carbonic acid esters (Zechner et al., 2012; Bezaire et al., 2009; Fredrikson et al., 1986). Highest expression of HSL mRNA and protein are observed in adipose tissue, and low expression is found in many other tissues, including muscle, testis, steroidogenic cells/tissues, pancreatic islets, and macrophages (Zechner et al., 2012; Holm et al., 2000).

The HSL enzyme activity is strongly induced by beta-adrenergic stimulation, while insulin has a strong inhibitory effect (Zechner et al., 2012; Lass et al., 2011). During beta-adrenergic stimulation, its activity is controlled by two different mechanisms: 1) Reversible phosphorylation of HSL at a number of serine residues by PKA, extracellular-signal-regulated kinase (ERK), glycogen synthase kinase-4, Ca^{2+}/calmodulin-dependent kinase II, and AMP-activated protein kinase (Watt and Hoy, 2012; Lass et al., 2011), 2) Translocation of HSL to the lipid droplets, which is regulated by perilipin-1 (Lass et al., 2011).

2.2.5.3 MGL

Monoglyceride lipase (MGL) involved in the final step of lipolysis, converting MAG into glycerol and free fatty acid. MGL is abundantly expressed among tissues, particularly in adipose tissue, kidney, and testis, which display high MGL mRNA expression (Lass et al., 2011). Study by Rakhshandehroo et al. (2007) demonstrated upregulation of MGL, along with other lipases (ATGL and HSL) transcripts, by PPARα stimulation in liver, thereby indicating PPARα as a negative regulator of steatosis. However, whether MGL is predominantly regulated at posttranscriptional or posttranslational level is not known as evidence of phosphorylation has yet to be determined (Labar et al., 2010).

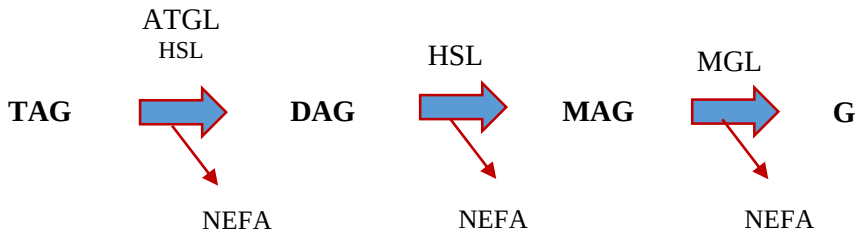


Figure 2.4: Schematic delineation of the coordinate breakdown of triacylglycerols. Abbreviations: ATGL, adipose triacylglycerol lipase; DAG, diacylglycerol; G, glycerol; HSL, hormone-sensitive lipase; MAG, monoacylglycerol; MGL, monoacylglycerol lipase; NEFA, non-esterified fatty acid; TAG, triacylglycerol (Adapted from Lass et al., 2011).

2.2.6 Intramuscular fat (IMF) development

The fattening phase always refer to the normal maturation phase, which involved changes in the lipid metabolism, while the growth phase, refers to the pre-fattening phase (Vernon, 1981). During the fattening phase, the nutrient for the skeletal and muscle growth were involved in the lipid anabolism activity, resulted in increased fat deposition (Vernon, 1980). This activity was in line early characterization with the developmental sequence of tissue deposition, which initiated with skeletal, muscle and fat (McMeekan, 1940; Hammond, 1932). In addition, the order of adipose tissue deposition in ruminant is

abdominal, intermuscular, subcutaneous, and finally intramuscular (Pethick et al., 2006).

Intramuscular adipose tissue, or intramuscular fat (IMF) or marbling, consists of accumulation of adipocytes, appearing as lipid droplets, located within the muscle, specifically in the perimysium (within intramyocellular and extramyocellular) in the muscle fibres. This is distinctly different to intermuscular fat, which is adipocytes and subsequent fat which occur in the spaces between the muscles) (Rahemi et al., 2015). Generally, the IMF deposition also includes uptake of fatty acid from the plasma in the form of NEFAs, or from fatty acid pools by the LPL action, followed by transportation into muscle mediated by fatty acid translocase (FAT/CD36). The glucose utilization also contributed for the acetyl-CoA production (building block for fatty acid synthesis) in the muscle (Tokach et al., 2010; Frayn et al., 2006). Meanwhile, the HSL mediated the oxidative activity of IMF (Frayn et al., 2006). IMF have been widely implicated to play an important role in meat quality. The amount of IMF positively correlated with sensory quality characteristics such as taste, flavour, firmness, juiciness, and tenderness of the meat, while lower IMF produces less tasty and dry meat (Hocquette et al., 2010; Pethick et al., 2006).

As reviewed by Hocquette et al. (2010), the deposition of IMF varies between species, including individual in given species, and between muscles and cuts. The activity and fibre type also influence the IMF level. Muscle with high muscularity and high glycolytic fibre types generally appear in reduced fat content in the muscle (Pethick et al., 2006). However, the development of IMF might also result from the balance between uptake, formation and breakdown of fat in the muscle, which involved an interrelation between intramuscular adipocytes and muscle fibre's metabolic activities (Hocquette et al., 2010). Hocquette and colleague also suggested that fat deposition might also resulted by the high fat turnover (Hocquette et al., 2003). The transdifferentiation of muscle cells and/or satellite cells into fat cells also might favour the IMF development in the muscle. However, this still needs to be elucidated (Hocquette et al., 2010).

Many studies reported that IMF content increases with feeding periods, until plateau (Tokach et al., 2010). During early growth, the rate of fat deposition is lower, while during maturation, the rate is higher than lean tissues, which might later increase IMF level (Pethick et al., 2004). IMF development can be represented using the S-shaped curve, which consists of early period (no change in IMF level), followed by linear growth and then reached the plateau (saturation of fat deposition at maturation). Adding to that, the curved might be shifted to the right after stimulation with certain metabolic modifiers, which stimulates muscle growth (Figure 2.5) (Pethick et al., 2006).

The identification of the key determinants in IMF development since early growth can be assessed by the expression of certain markers, which involved in the adipocyte differentiation such as PPARγ and SREBP-1, along with lipogenic enzyme such as ACC, FAT/CD-36 and LPL, which involved in lipid mobilization and uptake by the muscle (Hocquette et al., 2010; Hausman et al., 2009). However, as explained earlier, the fat turnover might be affecting IMF deposition and should be take into account. Study by Underwood et al. (2008) reported that key mediator in fat metabolism, AMP-activated protein kinase (AMPK) is negatively correlated with IMF level in LD muscle of beef cattle. This suggested that as AMPK promotes lipolysis or oxidation and impedes lipogenesis, the lower oxidative activity promotes fat deposition (Hocquette et al. 2010). By this reason, some researchers included blood analysis along with the enzyme activity studies to ascertain IMF development, noted that the different metabolic markers are included (Gondret et al, 2004b; 2004a). Review by Frayn et al. (2006) reported that the expression of FAS is small or undetectable, which suggested that the IMF might entirely developed from NEFAs.

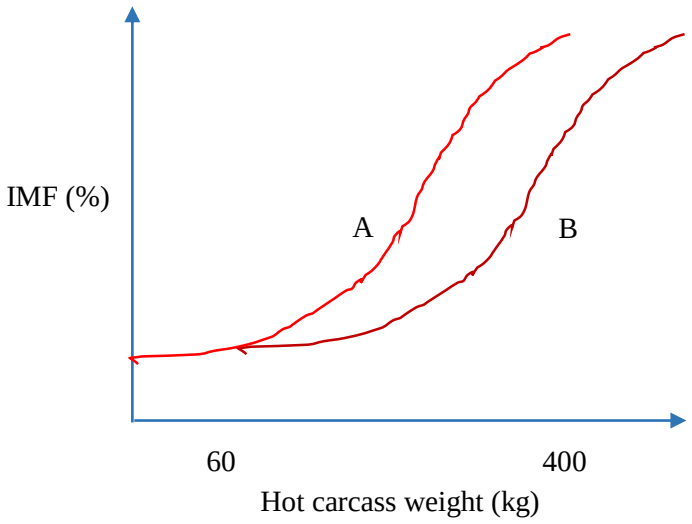


Figure 2.5: The IMF development. The S-shaped developmental curve hypothesized by Pethick et al. (2006), showing the IMF development at two different muscle weights, A = without metabolic modifiers, B= with metabolic modifiers (Adapted by Pethick et al., 2006).

2.2.7 Transdifferentiation - muscle cells to adipocytes

Transdifferentiation is an irreversible switching process of differentiated cell type to another, which both related in terms of ancestor-descendent relationship (Shen et al., 2004). It has been reported that transdifferentiation is likely to happen when there are any changes in the expression of the master regulator/switch genes that affect the developmental process, and can be induced by environmental changes, such as modification in cell culture media with certain inductive agents (Eguizabal et al., 2013; Shen et al., 2004).

As myoblasts and adipocytes are from the same origin (from mesoderm layer during embryogenic development), the transdifferentiation of myoblasts into adipocytes is possible and has been discussed widely, particularly in *in vitro* (Singh et al., 2007; Li et al., 2005), which commonly used C2C12 or G8 myoblast cell lines as a model (Grimaldi et al., 1997, Hu et al., 1995). Two key adipogenic transcription genes, PPARγ and C/EBPβ are involved in this process. In a study by Hu et al. (1995) using G8 myoblast cell lines, the co-expression and synergistic action of both genes suppressed the expression of myogenic

genes such as MyoD, myogenin, MRF4 and Myf5, and blocked further development into myotubes, induced the accumulation of lipid droplets-containing cells, and upregulation of adipogenic markers such as aP-2, adipsin, LPL, and PEPCK. In terms of intramuscular fat development, both genes were reportedly induced adipocyte numbers in the skeletal muscle of bovine (Torii et al., 1998) and pig (Poulos and Hausman, 2006).

The myoblast transdifferentiation into adipocytes can be induced by thiazolidinediones (TZD) family, such as rosiglitazone, pioglitazone, BRL49653, and unsaturated fatty acids (Grimaldi et al., 1997), which both are the natural and synthetic activators/ligands for the PPARγ receptor, respectively (Grygiel-Górniak, 2014). Both activators induced adipogenic markers expression, such as glycerophosphate dehydrogenase (GPDH), FAT/CD-36 and FABP, and suppressed myogenic markers expression, such as creatine kinase, myogenin and α-actin (Grimaldi et al., 1997). Treatment of C2C12 with TZDs inhibit MyHC and induced PPARγ and C/EBPα expressions, along with decreased fusion into myotube (Singh et al., 2007). TZDs also induced the expression of the GLUT-4 for the uptake of glucose, which is the substrate in the IMF development (Mukherjee et al. 2000).

The adipogenic media in cell culture, which is formulated with insulin, dexamethasone (DEX) and isobutylmethylxanthine (IBMX), along with the supplementation with TZDs, have been used in the myoblast transdifferentiation into adipocytes (Ryan et al., 2013; Singh et al., 2007). DEX induces C/EBPβ transcription, which is the factor for adipogenesis, IBMX stimulates cAMP levels and promotes adipogenesis, via cAMP response binding factor (CREB), while insulin acts through IGF-1R for lipid filing enhancement (Singh et al. 2007). Singh et al. (2007) also reported the exposure of adipogenic mixture and another TZDs, ciglitazone, to muscle satellite cells from porcine induced formation of lipid droplets-containing cells, and increase its number with increase incubation time, along with the expression of CEBP/α and PPARγ genes and activities.

2.3 METABOLIC MODIFIERS IN ANIMAL PRODUCTION

Metabolic modifiers are compounds that can be supplemented with feed, injected, or implanted to the animals, which will alter the biochemical changes in regulating nutrient use, targeted to the metabolism of protein (protein synthesis and protein turnover in skeletal muscle) and lipid (lipid turnover in adipose tissue), and milk synthesis and secretion in mammary gland, which consequently affect the efficiency of meat and milk production (CAST, 2005). A simple definition is that metabolic modifiers are an agent that affects how animals use absorbed nutrients. The metabolic modifiers in general, increase protein synthesis and decrease fat synthesis, thereby affecting the deposition of these two nutrients. A range of metabolic modifiers have been used to improve the rate of gain, the feed utilization efficiency, improve the carcass percentage, improve shelf-life of meat, improve meat's nutrient content and/or improve meat palatability (Gebre et al., 2012; Dikeman, 2007; 2003). Metabolic modifiers include naturally occurring growth hormone, and synthetic steroid hormone analogues and synthetic phenethanolamine compounds called β-adrenergic agonists.

2.4 GROWTH HORMONE (GH)

Growth hormone (GH), also known as a somatotropin (in the USA), is involved in growth control. GH is a part of cytokine superfamily, and a peptide hormone, primarily synthesized and expressed in the somatotroph cells of the anterior pituitary gland. GH secretion is regulated by two opposing neuroendocrine peptides, growth hormone releasing hormone (GHRH) and somatostatin, which stimulate and inhibit GH secretion, respectively (Vijayakumar et al., 2011; Velloso, 2008). Ghrelin, another peptide hormone is a potent GH secretagogue (GHS), and enhances GHRH stimulation and GH secretion from the pituitary, acting via its receptor (GHS-R) (Chaves et al., 2013; Lengyel, 2006). Ghrelin is primarily synthesized in hypothalamus and gastrointestinal tract (Lengyel, 2006; Kojima et al., 1999).

While GH can exert the metabolic effects by direct action with its own receptor, the growth hormone receptor (GHR), there is also an indirect action, which is via insulin-like growth factor-1 (IGF-1). The IGF-1 is mainly secreted from the liver by the action of GH. It plays an important role in negative feedback mechanism, which directly as well as indirectly has effects via stimulation of somatostatin, in inhibiting GH secretion (Figure 2.6) (Vijayakumar et al., 2011).

In the circulation, GH binds to the soluble form of GHR extracellular domain derived by alternate splicing or proteolytic cleavage, namely GH binding protein (GHBP) (Nam and Lobie, 2000). GHBPs have been suggested to involve in regulate the availability of the GH in the serum at all times and increasing stability of the GH, by increasing its half-life (Kopchick and Andry, 2000; Baumann et al., 1988).

Generally, GH plays an important role in the stimulation of bone and skeletal muscle growth, but it reduces or inhibits adipose tissue growth (Dikeman and Devine, 2014). The biological effects of GH can be classified either somatogenic or metabolic, with the somatogenic effects being mediated by the IGF-I causing the stimulation of the cell proliferation. In contrast, many of the metabolic effects are via direct action of GH on a variety of tissues with the metabolism of all macro nutrient classes (carbohydrate, lipid, and protein) being affected — along with minerals. These effects alter nutrient partitioning and thus, play a key role in growth performance or milk yield (Etherton and Bauman, 1998).

2.4.1 GH mechanism of action

The signal of reduced or excess food intake has to be translated into altered expression levels of lipogenic genes. In terms of fasting, this is associated with a decrease in plasma glucose and an increase in plasma-free fatty acids. Plasma glucose concentrations stimulate lipogenesis via several mechanisms. First, glucose itself is a substrate for lipogenesis. By being glycolytically converted to acetyl-CoA, glucose promotes fatty acid synthesis. Secondly, glucose induces the expression of lipogenic genes, the mechanisms of which are explained below.

Finally, glucose increases lipogenesis by stimulating the release of insulin and inhibiting the release of glucagon from the pancreas (Kersten, 2001).

In GH-treated animals, the changes in lipid metabolism can be observed to redirecting nutrients to be deposited in lean tissue (repartitioning agent) and to improve productive efficiency (reduce proportion of nutrients used for fat synthesis). Accretion of lipid in adipose tissue is a function of the relative rates of lipogenesis and lipolysis. The effects of lipid synthesis being a major importance if animals are in positive energy balance, whereas the effects of lipolysis predominate when animals are at energy balance near to zero or negative (Etherton and Bauman, 1998). When animals are in negative energy balance, rates of lipogenesis are already low and GH treatment affects adipose tissue by increasing rates of lipid mobilization, decreasing body fat, chronically elevating of non-esterified fatty acids (NEFAs), and increasing fat percent as well as the proportion of long-chain fatty acids found in milk. In contrast, when animals are in positive energy balance, GH causes a reduction in rates of lipid synthesis, like in growing animals, however, there is no change in NEFA irreversible loss rate, milk fat percent, or milk fatty acid composition (National Research Council, 1994).

In terms of gene expressions, the GH administration reduces the fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) activity in adipose tissue of growing pigs and *in vitro* cultures of cow adipose tissue (Lanna et al., 1995). The fact that GH reduces FAS and ACC suggests that GH affects lipid metabolism by altering transcription of these key metabolic genes.

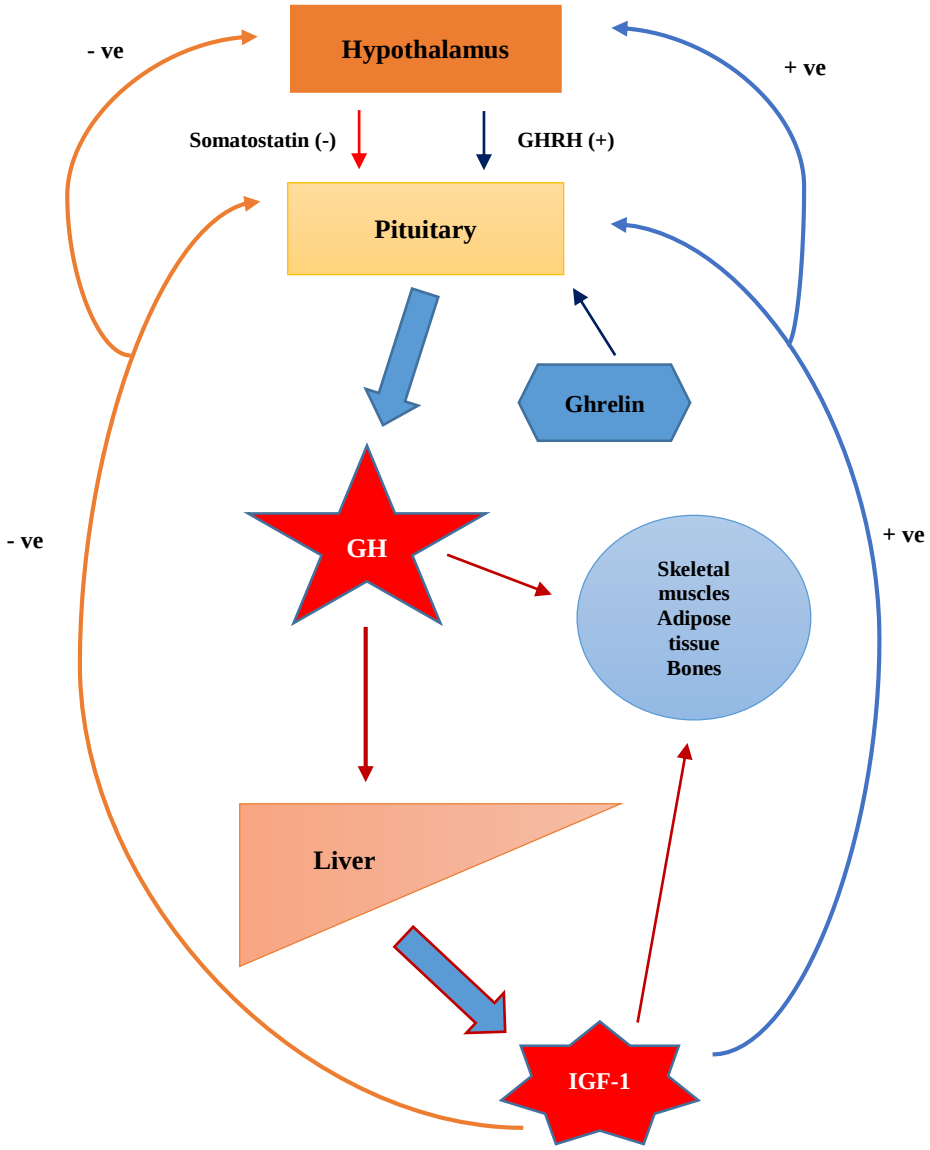


Figure 2.6: Regulation of GH secretion. GH secretion is controlled by two hypothalamic peptides: the stimulatory GHRH and the inhibitory somatostatin. GH, secreted by pituitary somatotroph cells, stimulates the production of insulin-like growth factor I (IGF-I) in liver stimulates growth in the peripheral tissues. IGF-I and GH are believed to feed back (+ve or –ve), at the hypothalamus and pituitary as illustrated, to modulate GH secretion.

2.4.2 IGF-1

GH stimulates upregulation of mRNA and protein level of insulin-like growth factor 1 (IGF-1, also known as somatomedin-C) in the liver. Apart the ability to act as endocrine/circulating IGF-1, it also plays an important role as autocrine (secreted by the cells itself) or paracrine (induction by the adjacent cells) growth factors in other tissues (Vijayakumar et al., 2011; Velloso, 2008; Philippou et

al., 2007). The serum IGF-1 level, produced by the liver acts as suitable marker in GH regulation (Buckway et al., 2001).

As described above, the IGF-1 mediates indirect GH action according to somatomedin hypothesis, which states that GH indirectly regulates somatic growth via controlling the production of hepatic IGF-1, which is then transported through the circulation, effecting the target organs/tissues (Wang et al., 2013; Vijayakumar et al., 2010; Laron, 2001). However, in addition to the liver, the IGF-1 level in the circulation is also contributed by the extra-hepatic tissues, skeletal muscle and adipose tissue (Vijayakumar et al., 2011; Philippou et al., 2007).

The structural homologous of IGF-1 with insulin explains the ability of IGF-1 to bind to IR, but with lower affinity that its own receptor, IGF-1R (Pandini et al., 2002; Laron, 2001). In the circulation, most of the IGFs bound to the binding protein, IGF-binding proteins (IGFBPs). Most IGF-1 formed ternary complex with IGFBF-3, for the localization of the IGF-1 to the organs/tissues, and acid labile subunit (ALS), for half-life stability of IGF-1 (Velloso, 2008; Laron, 2001). IGFBPs also modulate IGFs action on the tissues as their affinity for IGFs is higher than the receptors (Velloso, 2008).

2.4.3 Signalling pathway by GH and IGF-1

The action of GH depends on its interaction through the GHR on a target tissue. GHR belongs to type 1 cytokine/hematopoietin receptor family. The activation of GH, at the extracellular domain (ECD) of two GHR receptors result in the dimerization of both GHR molecules, which lead to the conformational changes in the receptors and activates cytosolic kinase associated with GHR, Janus kinase 2 (JAK2) in the intracellular domain (ICD), which in turn transphosphorylate the adjacent JAK2 molecules. The activated JAK2 molecules phosphorylate tyrosine residues on GHR, which in turn recruits other signalling molecules called signal transducers and activators of transcription (STATs), particularly STAT5. The STATs molecules dimerize, translocate to the nucleus, and bind to

DNA to stimulate target gene transcription, including IGF-1 (Figure 2.7) (Vijayakumar et al., 2011; dos Santos et al., 2001; Kopchick and Andry, 2000).

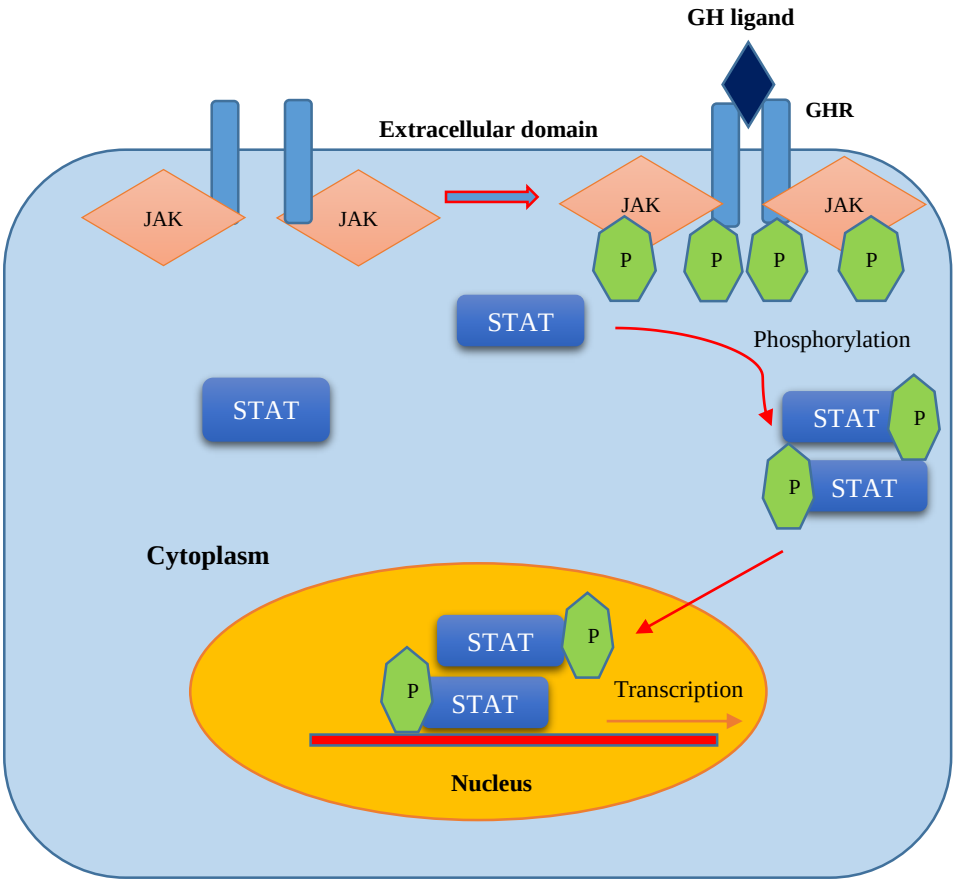


Figure 2.7: Simplified diagram of GH signalling pathway via JAK/STAT pathway. GH ligand to cytokine receptor, GHR, dimerizes and activates the adjacent JAK kinase, which in turn phosphorylates STAT proteins. STATs form a heterodimer before translocate to the nucleus for the transcription of gene expression.

The action of GH signalling can be inhibited by the action of inhibitors such as suppressor of cytokine signalling (SOCS) protein family, which downregulates JAK2 activity, and protein tyrosine phosphatase (PTPs), which involve in the termination of the GHR signal cascade (Herrington and Carter-Su, 2001; Kopchick and Andry, 2000).

Meanwhile, for IGF-1, it binds to its receptor, IGF-1 receptor, (IGF-1R), a transmembrane protein kinase receptor, induced autophosphorylation and activated receptors recruits the signalling proteins such as insulin receptor substrate-1 (IRS-1), which in turn activates two signalling mechanisms,

phosphatidylinositol-triphosphate kinase (PI3K)/Akt mechanism and mitogen activated protein kinase (MAPK) mechanisms (Vijayakumar et al., 2010; Velloso, 2008).

For the first pathway, the activation of PI3K resulted subsequent activation of serine/threonine specific protein kinase (Akt; or protein kinase B), which then leads to activation of the kinase mammalian target of rapamycin (mTOR). The activation of mTOR leads to the stimulation of protein synthesis by inducing p70 S6 kinase (P70S6K), the positive regulator for protein translation, and inhibiting 4E-BP (also named as phosphorylated heat- and acid-stable protein 1(PHAS-1)) phosphorylation, which prevents the release of negative regulator of protein initiation factor, eIF-4E. The second Akt downstream is glycogen synthase kinase 3β (GSK3β), which is inactivated by the activated Akt. The inhibition of GSK3β, in turn phosphorylates and inhibits protein translation regulator, eIF-2B protein for the protein synthesis. In addition to protein synthesis, the activated Akt also involved in the inhibition of protein breakdown by the prevention of the nuclear translocation of the transcription factor FOXO, which is modulate the expression of two muscle atrophy ubiquitin ligases, namely muscle ring finger1 (MuRF1) and muscle atrophy F-box (MAFbx) (Figure 2.8). This pathway has been widely accepted to be involved in the skeletal muscle hypertrophy (Shavlakadze et al., 2010; Velloso, 2008; Nader, 2005; Glass, 2005; Machida and Booth, 2004; Glass, 2003).

In the second pathway, the ligand of the IGF-1 with IGF-1R initiates the Shc phosphorylation, and in turn the formation of the guanine-nucleotide exchange factor (GEF) complex, Grb2/Sos. These complex binds to the small G protein, Ras, resulting in the replaced of GDP (inactive state) to GTP (active state). The activated Ras (GTP-bound) activates the kinase, Raf (or mitogen-activated protein kinase kinase kinase (MAPK3K)) and further activates second downstream kinase, MEK (or MAPK2K), and subsequently activates final downstream kinase, ERK (or MAPK). The activation of MAPK, by double phosphorylation of its threonine and tyrosine residues, and induces its translocation into the nucleus for the regulation of transcriptional factors (Guntur and Rosen, 2013; McCain, 2013; Siddle, 2011).

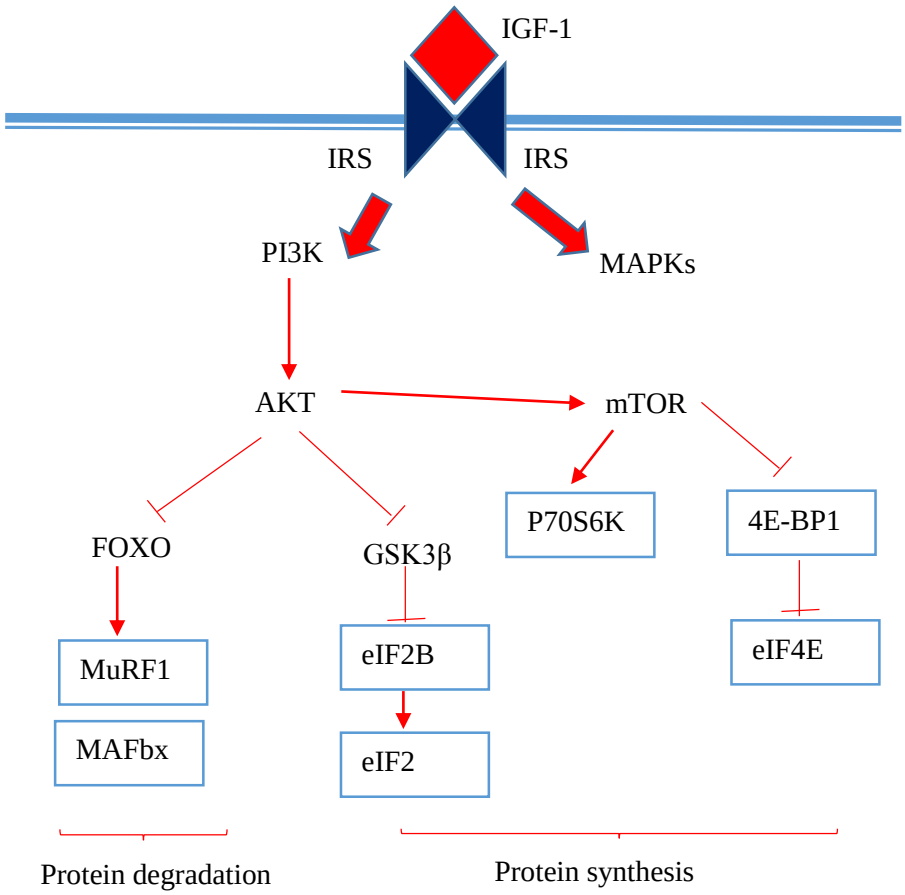


Figure 2.8: Simplified diagram of IGF-1 signalling pathway. IGF-1 acts on IRS, activates two mechanism; PI3K/AKT pathway, which involved in protein metabolism by the activity of FOXO transcription factors; and MAPKs pathway, which also regulates the activation of the transcriptional factors, after translocation into the nucleus (Adapted from Velloso, 2008)

2.4.4 Biological effects of IGF-1

IGF-1, primarily secreted by the liver, is widely considered as a potent growth factor, which mediated the effects of GH in human and animals. It has been involved in the skeletal muscle development or hypertrophy (Velloso, 2008). Additionally, the effect of IGF-1 on muscle regeneration via satellite cell proliferation have been reported (Machida and Booth, 2004), which might contribute for the salvage of aging-related muscle atrophy. The stimulation of lean tissue development by the IGF-1 have been observed, with plasma IGF-1 level was positively correlated with the rate of growth (Owens et al., 1999). IGF-1 plays a role in respiratory development, as the absence of IGF-1R exhibit

impaired diaphragm development and intercostal muscles, resulted in respiratory failure (Accili et al., 1999).

2.4.5 Effects of GH on growth

GH-treated animals showed increased in growth rate and improvement in feed efficiency (body weight gain per feed consumed) and protein deposition (muscle development) (Flint et al., 2003; Oscarsson et al., 1999). GH also has the effect of repartitioning nutrients agent between muscle and adipose tissue, partitioning nutrients to muscle, and reduced their utilization by adipose tissue. GH treatment also reduces hepatic and peripheral insulin sensitivity in human, which subsequently reduces insulin regulated events such as glucose transport, expression of lipogenic enzymes activities and lipogenesis (Møller et al., 2009; Flint et al., 2003). This event also affected the nutrient repartitioning effect mentioned above. GH-knockout mice showed impaired growth and skeletal development, and respiratory failure caused by impaired development of diaphragm and intercostal muscles in IGF-1R-knockout mice (Laron, 2001). In GHR-knockout mice, the mice present postnatal growth retardation including increase GH and decrease circulating IGF-1 levels, indicating a complete GH resistance (Sotiropoulos et al., 2006).

2.4.6 Effects of GH in adipose tissue

2.4.6.1 Effects of GH in lipolysis

GH has been known as lipolytic agent. Earlier, the removal of pituitary glands (hypophysectomy) in rats resulted in increased body fat and decreased lean mass, which associated with GH deprivation (Scow and Chernick, 1960; Li et al., 1949). In human, the increase in lipolytic activity has been shown via pulsatile secretion rather than constant GH delivery (Cersosimo et al., 1996). The adipose tissue in rat and human also have been observed to undergone lipolysis with GH treatment, resulted in increased plasma NEFAs, which is the marker in lipolysis by the triglyceride catabolism (Chaves et al., 2013). A study by Sakharova et al. (2008) showed that the increase in the GH secretion stimulated by fasting, was

also accompanied by the significant increase of lipolysis. In the culture media of primary adipocytes treated with GH, the amount of glycerol, another lipolytic marker, was significantly higher than in media of untreated adipocytes (Wabitsch et al., 1996). The accumulation of glycerol also has been observed in neonatal and growing pig adipose tissue after 24-hour treatment with porcine somatotropin (pST) (Wang et al., 1999). Administration of GH induces lipolysis in the adipose tissue, is thought to be mediated by the action of hormone-sensitive lipase (HSL) stimulated via the cAMP/PKA pathway, which is more pronounced in the visceral compared to subcutaneous depots (Chaves et al., 2013; Lonnqvist et al., 1993). The *in vitro* study of chronic GH treatment using 3T3-F442A adipocyte cells showed increase in HSL activity and glycerol release, together with dose-dependent effect of GH in inducing lipolysis (Dietz and Schwartz, 1991).

2.4.6.2 Effects of GH in lipogenesis

GH may modulate the deposition of lipid by reducing fatty acid biosynthesis, especially in growing animals (Chaves et al., 2013). In growing pigs, the treatment by dose-dependent porcine somatotropin (pST) for 30-60 days resulted in decrease lipid deposition, which was due to reduce glucose incorporation into lipogenesis, while lipolysis was unaffected (Etherton et al., 1993). The GH also decreased lipogenesis and lipoprotein lipase (LPL) activity in vitro of neonatal swine adipose tissue culture (Wang et al., 1999). Since the fatty acids can be derived from hydrolysis of circulating chylomicron and lipoprotein triglycerides by LPL, for adipose tissue uptake for storage (Goldberg et al., 2009; Weinstock et al., 1997), the reduction in LPL activity might affect the lipogenesis in adipose tissue. The effects of GH also have been observed on the mRNA expression and activity of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which both are the key lipogenic enzymes *in vivo* and *in vitro,* both in ruminant and non-ruminant animal cells. Daily pST treatment downregulates ACC mRNA expression and activity by up to 50% (Liu et al., 1994), meanwhile reduced both FAS activity and mRNA expressions were observed in the bovine somatotropin (bST) treatment in lactating cows, accompanied with decreased in ACC activity

(Lanna et al., 1995), as well as in 3T3-F442A adipocytes (Yin et al., 1998) and pST treatment in pigs (Donkin et al., 1996).

2.4.6.3 Effects of GH in adipogenesis

GH treatment is also involved in the regulation of differentiation of adipocyte (adipogenesis). The modulation of the GH in the regulation of adipogenesis is mainly via its receptors (GHR) and GHBP. During adipogenesis, the GHR mRNA and protein expression are also regulated, which both upregulated in mature adipocytes compared to preadipocytes (Nam and Lobie, 2000). The GHBP also play a role in adipogenesis. The equal binding affinity of GH and maintaining their constant ratio are influenced by both GHR and GHBP expression on the adipocyte surface, which subsequently affecting adipogenesis (Nam and Lobie, 2000). The GHBP and GHR compete each other for the preadipocyte surface ligand, inhibits its differentiation to mature adipocyte, meanwhile intracellular GHBP has been shown to act as a transcriptional enhancer for STAT5-mediated pathway for adipocyte differentiation (Nam and Lobie, 2000).

In regard to the type of preadipocytes, there are different effects of GH dependent on whether treated on clonal (such as 3T3-L1, 3T3-F442A and Ob1771) or primary (obtained directly from animal or human adipose tissue) cell lines. In clonal cell lines, GH has been demonstrated to inhibit the proliferation and induce the differentiation of preadipocytes, which can be categorized as antimitogenic (Nam and Lobie, 2000). In the 3T3-F442A preadipocyte cell line, GH modulates the cell division by arresting the cell cycle with the associated downregulating the cyclin-D1 expression, one of the modulators in the cell cycle (Tang et al., 1995). Moreover, the treatment of the 3T3-F442A preadipocyte cell lines with human GH induced the expression of C/EBPβ and C/EBPδ, which are both implicated in the stimulation of adipocyte differentiation (Clarkson et al., 1995). Meanwhile, Doglio et al. (1986) demonstrated GH upregulated the expression of differentiation-dependent genes such as glycerol-3-phosphate dehydrogenase and p422 protein in Ob1771 preadipocyte cell line. The expression of c-fos and c-jun was also stimulated following the GH treatment,

which related to adipocyte differentiation (Sumatran et al., 1992; Doglio et al., 1989).

In contrast with clonal cell lines, GH induces the proliferation, whilst attenuating the differentiation of primary cell lines into adipocytes. In primary porcine preadipocyte cell line, the human growth hormone (hGH) inhibited the development of fat cell clusters, and decreased in the glycerol-3-phosphate dehydrogenase activity level, indicative for the impairment in differentiation, by dose dependent manner (Hausman and Martin, 1989). A study by Hansen et al. (1998) using primary rat preadipocyte cell line demonstrated the downregulation of late markers for adipocyte differentiation, including FAS, aP2, and HSL, while modest reduction in PPARγ expression. This indicating the inhibition of GH on PPARγ-induced transcription, which affect adipocyte differentiation. They also demonstrated that GH prevented the downregulation of preadipocyte factor-1 (PREF-1), which may arrest the differentiation of adipocytes.

As mentioned earlier, the regulation of GH is mediated via JAK/STAT pathway. The activated STAT molecules, particularly STAT5, enhanced the expression of C/EBPβ, C/EBPδ, and PPARγ, as demonstrated in the 3T3-L1 preadipocyte cell line (Kawai et al., 2007), which indicative of direct adipogenesis induction by GH.

2.4.7 Effects of GH in liver

In terms of lipid metabolism, GH has been demonstrated to induce the expression of lipoprotein lipase (LPL) and/or hepatic lipase (HL), which are both implicated in the lipid flux and distribution in the liver (Oscarsson et al., 1999). Study by Neve et al. (1997) demonstrated that the reduced expression of HL in the hypothyroid rat can be normalized by the GH administration, which restore and stabilize the HL mRNA levels, and stimulates transcription. The expression of hepatic LPL was elevated in the liver of the mice overexpressing bovine growth hormone (bGH) (Wang et al., 2007). Furthermore, GH also involved in the phosphorylation and transcription of the sterol regulatory element-binding

transcription factor 1 (SREBP-1), particularly SREBP-1a, which is the modulator in lipid production, in the human liver HepG2 cell line, via Erk-MAPK signalling pathway (Kotzka et al., 2010).

As reviewed by Vijayakumar et al. (2010), the role of GH treatment in the hepatic lipid storage is mediated via lipolytic or oxidative inhibition of intrahepatic lipid, or stimulation of lipogenesis. This was indicated by the observation in the bGH transgenic mice of the downregulation of lipolytic genes, hepatic HSL expression, along with downregulation lipid oxidative genes expression, such as acetyl-CoA oxidase (ACO-1) and PPARα, as well as enhanced expression of lipogenic gene such as ACCα (Wang et al., 2007; Olsson et al., 2003).

2.4.8 Effects of GH in skeletal muscle

GH stimulates the expression of LPL, which resulted in increased uptake of free fatty acid (FFA) into skeletal muscle. In hypophysectomised rats and GH deficient-adult, the GH treatment induces LPL expression in skeletal muscle (Khalfallah et al., 2001; Oscarsson et al., 1999). The replacement therapy of the GH-deficient adult leads to an upregulation of the muscle HSL mRNA expression and seems to increase muscle fatty acid-binding protein (FABP3) mRNA expression, indicative for the increase of free fatty acids mobilization and uptake within the muscle (Trepp et al., 2008). They also demonstrated the increase in skeletal muscle IGF-1 mRNA indicating the effects was also mediated by locally synthesized IGF-1 (Trepp et al., 2008). Moreover, study by Kim et al. (2008) demonstrated that GH treatment to the GH-deficient lit/lit (GHRH receptors inactivation by mutation) mice after 4 hours, resulted in further increase of the PPARδ and its mRNA expression, suggesting that lipid oxidation regulation takes place acutely. In the same experiment, the Forkhead box protein O1 (FoxO1) mRNA in GH-deficient lit/lit mice was also increased by the GH treatment. This could be due to the suppression of P13K/Akt pathway which leads to the activation of proteolytic enzyme and reduction in muscle mass in GH-deficient state.

Regarding GHR deletion, the primary cultures derived from GHR-knockout mice displayed postnatal reduced cross-sectional area (CSA) of the myofibre or myofibre atrophy, and stimulate transition from type I (oxidative) to type II (glycolytic) fibres, indicating GH involved in the of type I fibre phenotype determination (Sotiropoulos et al., 2006). Sotiropoulos et al. (2006) also demonstrated that GH independently modulate muscle cell extension at the later phase of differentiation. This was not mediated by IGF-1, as GHR activation did not rely on changes in IGF-1. GH also involved in the activation of satellite cells by stimulating the expression of mechano-growth factors (MGFs) and IGF-IEa (splice IGF-1 cloned), with the parallel stimulation of MyoD expression, which suggests the satellite cells proliferation (Imanaka et al., 2008). Moreover, the GH treatment inhibits myostatin expression, which is a myogenesis inhibitor, suggesting that GH modulates myoblast regeneration (Liu et al., 2003).

As a summary, the effect of GH in target tissues particularly in lipid metabolism are complex. The GHR activation stimulates the phosphorylation and transcription of certain enzymes, which involve in transport and catabolic activity of the lipid, promoting the increase in lipid mobilization and reduce lipid deposition.

2.5 BETA-ADRENERGIC AGONISTS (BA)

Beta-adrenergic agonists (BAs), also known as beta-agonists, are natural or synthetic compound, which are structural analogs of the natural catecholamines hormones adrenaline and noradrenaline, and categorized as phenethanolamines and the synthetic catecholamines, which are compounds such as ractopamine hydrochloride and zilpaterol hydrochloride (CAST, 2005; National Research Council, 1994). Several beta-agonists are used therapeutically in human and animal medicine for the specific effects on smooth muscle and as antiobesity agents (CAST, 2005). In the USA, ractopamine hydrochloride (RH) and zilpaterol hydrochloride (ZH) are both approved as growth promoters for swine and beef cattle, respectively. These agents are orally active and efficacious at 5-

30 parts per million (ppm) of feed when fed for a short period time (28-42 days) near the end of the finishing period just before slaughter (Johnson et al., 2014; CAST, 2005).

2.5.1 BA receptor subtypes

The adrenergic receptors are members of large G protein-coupled receptors (GPCRs) superfamily, the transmembrane protein, which stimulate intracellular G proteins upon ligand with endogenous catecholamines. The adrenergic receptors are divided into two different types: α-and β-adrenergic receptors. The α-adrenergic receptor (α-AR) is further divided into two different subtypes: α_1- and α_2-AR, while β-adrenergic receptor (β-AR) is divided into three subtypes: β_1-, β_2-, and β_3-AR (Louis et al., 2000). According to their regulation upon the ligand, the α_1- and α_2-AR are responded to two other G proteins, Gq and Gi, respectively. Gi prevents adenylyl cyclase activation, which in turn inhibits cAMP and PKA stimulation, and Gq activates phospholipase-C (PLC) and phosphatidylinositol and diacylglycerol (IP3/DAG) signalling pathway. In contrast, β-ARs act on the stimulatory G (Gs) protein, which subsequently activate the signalling cascade involving the activation of adenylate cyclase and cAMP/PKA signalling pathway which will be discussed further below (Johnson et al., 2014; Lodish et al., 2000).

The distribution of β-ARs have been identified to be present almost on every mammalian cell surface (Mersmann, 1998). The prevalence of β_1-, β_2-, and β_3-AR distributions are varies between tissues and species (Mersmann, 1998). β_1-AR predominantly found in heart of rats and pigs, as well as lung and liver in human (Johnson et al., 2014). β_2-AR, have been exclusively found on the skeletal muscle and adipose tissue in bovine, as well as on the bronchioles in the lung, in rats (Johnson et al., 2014; Hagström-Toft et al., 1998; Sillence and Matthews, 1994). The β_3-AR is abundant in white and brown adipose tissue in human, as well as in heart, mammary and placental artery, and brain, while in cattle, it is predominantly expressed in adipose tissues, mammary glands, and gastrointestinal tracts (Wu et al., 2011).

Moreover, the stage of the cell differentiation also influences the composition of β-AR subtypes, however the mechanism for this is poorly understood. A study by Fève et al. (1991) demonstrated that the undifferentiated primary preadipocytes 3T3-F442A from rodent, is predominantly expresses β_1-AR mRNA, however β_3-AR mRNA increase as these cells differentiates. In contrast, in clonal 3T3-L1 cell lines, there was no change in β_1-AR or β_2-AR mRNA at the immature state, but β_2-AR mRNA expression increased during differentiation (Guest et al., 1990). The treatment with dexamethasone also stimulates the accumulation of β_2-AR during differentiation (Lai et al., 1982). These *in vitro* studies might indicate the β-ARs are altered by growth, and corresponded to the animal age (Mersmann, 2002).

The function of these receptors also can be explained using gene targeted mice. In double β_1/β_2-AR-knockout mice, the ionotropic and chronotropic effects were abolished (Rohrer et al., 1999). The β_2-AR-knockout mice displayed a lower respiratory exchange ratio (RER: oxygen consumption to carbon dioxide production ratio), indicative for higher oxygen consumption, which is indicative of fatty acids being used as a substrate, and associated reduce body fat content (Chruscinski et al., 1999). Meanwhile, β_3-AR-knockout mice showed a moderately increased body fat, indicating the role in energy balance regulation (Susulic et al., 1995).

2.5.2 BA mechanism of action

BA exhibit repartitioning effects on growth and metabolism of skeletal muscle and adipose tissue. They act directly through β-ARs on skeletal muscle and adipose cell membranes. Subsequently they generate signals that control metabolic activities in the cells such as the stimulation of glycogenolysis and production of lactate in the skeletal muscle, and stimulation lipolysis in the mammalian adipose tissue (Dikeman and Devine, 2014; National Research Council, 1994).

In the fat cells, the biochemical signals that have been activated by BA will presumably activate several enzymes and lead to the decreased rates of lipogenesis (lipid synthesis and storage) and to the increased lipolysis (lipid mobilization in cell) (CAST, 2005). In skeletal muscle, there is hypertrophy and/or increased cross-sectional area of individual muscles rather than hyperplasia (Johnson et al., 2014). The effect of the treatment of BA is the regulation of protein accretion resulting an increase in protein synthesis and decrease in protein degradation in muscle fibres (Fiems, 1987).

2.5.3 Signalling pathway of BA

As described earlier, β_2-ARs are primarily expressed in the skeletal muscle, adipose tissue and lung. The activation of the β_2-ARs by the ligand, such as adrenaline, stimulate the conformational changes and the interaction of the receptor to a trimeric stimulatory (Gs protein) (which is comprising G_α-, G_β-, and G_γ-subunits). The release of guanosine diphosphate (GDP) and binding of guanosine triphosphate (GTP) of the G_α-subunit stimulates its separation from the G_β- and G_γ-subunits dimer (Barisione et al., 2010). The activated G_α-subunit leads to the stimulation of the adenylyl cyclase (AC), while G_β- and G_γ-subunits dimer, activate other signals (Johnson, 2006). Upon the binding with the AC, the energy from hydrolysis of the terminal phosphate from GTP is transferred and subsequently activated the AC. This process of the conversion from GTP to GDP by the GTPase activity causes the G_α-subunit to dissociate from the activated-AC and re-associate to the G-protein. The activated-AC, stimulates an increase in intracellular concentrations of second messenger, cyclic adenosine monophosphate (cAMP) by adenosine triphosphate (ATP) conversion, which then binds to the regulatory subunit and activates protein kinase A (PKA). The release of the PKA catalytic subunit then stimulates the phosphorylation certain proteins such as hormone sensitive lipase (HSL) (for lipolysis), acetyl-CoA carboxylase (ACC) (for lipogenesis). It also phosphorylates the cellular transcription factor, cAMP response element binding (CREB) protein, affecting its binding to DNA sequence and modulate expression of certain genes. (Figure 2.9) (Barisione et al., 2010; Johnson, 2006; Mayr and Montminy, 2001).

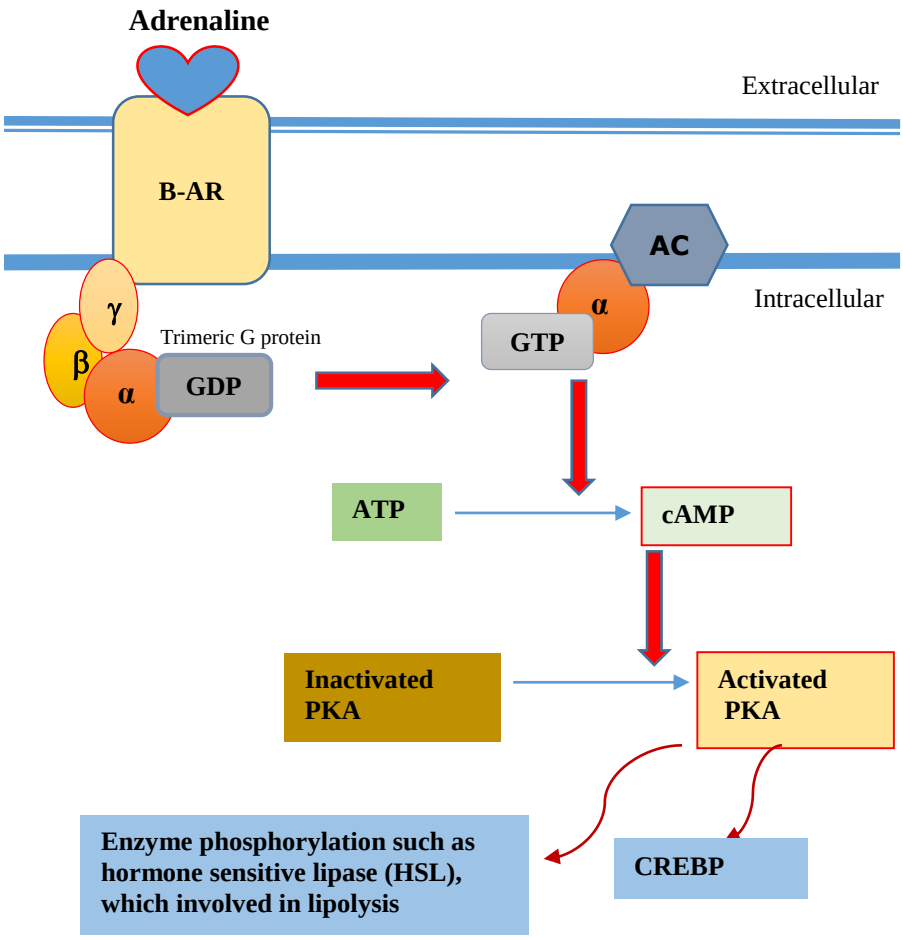


Figure 2.9: Simplified diagram of BA signalling pathway. Adrenaline ligands with the B-AR, activated the trimeric G protein. GTP binding on Gα stimulates its dissociation and activates adenylyl cyclase (AC), which in turn stimulates an increase in intracellular cAMP by ATP conversion, which is then activates PKA. The release of the PKA catalytic subunit then stimulates the phosphorylation certain proteins such as hormone sensitive lipase (HSL) (for lipolysis), acetyl-Coa carboxylase (ACC) (for lipogenesis), as well as phosphorylation cAMP response element binding (CREB) protein to modulate certain gene expression.

2.5.4 Effects of BA in skeletal muscle

2.5.4.1 Muscle hypertrophy

BA has been considered as the most potent agent in stimulating muscle hypertrophy in several species, as reviewed by Lynch and Ryall (2008). The hypertrophy can be seen as increased in cross-sectional area and mass of the muscles in ruminants (Johnson et al., 2014). Hinkle et al. (2002) demonstrated no observation of hypertrophy on the muscles in mice lacking β_2-AR compared

with wild type and β_1-AR-knockout mice after treated with clenbuterol, suggesting that the muscle hypertrophy is modulated through by β_2-AR. This observation is in line with the higher density of β_2-AR in skeletal muscle (Watson-Wright and Wilkinson, 1986). The treatment of selective beta-antagonist, ICI118551 in the combination with the BA clenbuterol attenuated the anabolic effect of the latter, suggesting the BA-mediated muscle hypertrophy (Sillence et al., 1991).

The hypertrophy effect of the BA appears to be instigated by the upregulation of protein synthesis rate and/or downregulation in protein degradation rate via the activation of Akt/mTOR pathway (Johnson et al., 2014; Lynch and Ryall, 2008; Kim and Sainz, 1992). Study by del Barrio et al. (1995) demonstrated in lamb treated with salbutamol, there was an increase in protein content and a reduction in protein degradation rate. Additionally, there was also stimulation of gene expression related to the translation initiators such as eukaryotic initiation factor 2b (Eif2b) and elongation initiation factor 5 (Eif5) (Spurlock et al., 2006), indicative for protein synthesis. Meanwhile, cimaterol treatment in lambs also resulted in the increased in RNA concentration in *semitendinosus* muscle, and increased RNA to DNA ratio, which implicated an increase in protein synthesis (Beermann et al., 1987), suggestive for the muscle hypertrophy. A review by Johnson et al. (2014) also described the increase in myofibrillar protein gene transcription, such as myosin heavy chain protein, upon BA treatment, which may contribute to the increased in myofibrillar protein synthesis and muscle hypertrophy.

As explained earlier, BA stimulation, via the action of cAMP, also influenced the intracellular Ca^{2+} concentration, which might be implicated in the stimulation of muscle proteolysis and inhibition of protein synthesis. The increase in intracellular Ca^{2+} has been proposed to stimulate protease activity, which would be expected to result in greater net muscle proteolysis (Turner et al., 1988). However, in contrast, BA-induced hypertrophy has been also observed to be associated with increased calpastatin activity, which inhibits calpain proteolytic activity, thereby potentially reducing proteolytic capacity (Parr et al., 1992). For example, the BA, L-644,969, was shown to increase the activity of the calpain

inhibitor, calpastatin (Johnson et al., 2014). Any amino acids released from the proteolysis would be expected to be reutilized to form new proteins. Alternatively, these amino acids could be used as a substrate for gluconeogenesis (glucogenic amino acids) or the production of ketone bodies (ketogenic amino acids), as well as synthesis of new specialized products such as porphyrins, neurotransmitter, hormones, purines and pyrimidines (Ferrier, 2014).

The hypertrophic effects of BA were also demonstrated according to the muscle fibres. There are three types of muscle fibres, which are type I fibre (slow twitch, oxidative), Type IIa fibre (fast twitch, oxidative), and Type IIb fibre (fast twitch, glycolytic). The histochemical studies by Zeman et al. (1988) demonstrated the fast twitch fibres in the soleus undergo hypertrophy and increase fast to slow fibre ratio upon clenbuterol administration, indicative the 'fast-like' behaviour. In addition, the Type II Myosin Heavy Chain (MyHC) protein expression also elevated in the *soleus* (slow, oxidative fibre) after clenbuterol treatment, as well as inhibition of Type I MyHC protein expression in *extensor digitorum longus* (EDL) (fast, glycolytic fibre), suggesting the transition of the slow to fast fibres (Rajab et al., 2000). The fibre transition also occurred within the same subtype as demonstrated by Maltin et al. (1989), who observed a reduction in fast oxidative/glycolytic fibres and increase fast glycolytic fibres in EDL.

The BA effect of muscle hypertrophic is much more consistent in fast-twitch muscle, especially in increasing cross-sectional area in the muscle compared with slow-twitch muscle. This might be explained with the upregulation of the nuclear receptor NR4A3 mRNA and protein expression in the fast-twitch muscle (Kawasaki et al., 2011) upon BA treatment, which can repress the expression of myostatin, the negative regulator in muscle mass (Paeren et al., 2006). The role of clenbuterol in ubiquitin-proteosome inhibition in fast-twitch muscle, but not in slow twitch muscle, also might stimulate hypertrophic effect of fast-twitch muscle, and reduced muscle atrophy (Yimlamai et al., 2005).

2.5.4.2 Intramuscular fat lipolysis

It has been reported that skeletal muscle comprises a considerable amount of both triacylglycerides and free fatty acids (Gorski, 1992). The accumulation of intracellular triacylglycerol in the form of lipid droplets plays an important role in maintaining intracellular lipid homeostasis, as well as acting as a major source as metabolic fuel in the skeletal muscle cells, via lipid oxidation (Morales et al., 2017). This component, which is referred to as intramuscular fat (IMF), influences the meat quality, and numerous studies have confirmed this relationship (Hocquette et al., 2010; van Laack et al., 2001).

The effect of BA on meat quality is to reduce meat tenderness (Dikeman, 2007), which might be, in part, caused by increased in lipolysis. The BA stimulation induced the lipolysis is demonstrated by an increase of skeletal muscle glycerol level (lipolysis end product) demonstrated by BA injection by microdialysis technique in human *gastrocnemius* muscle (Hagström-Toft et al., 1998). In addition, the non-selective β-AR antagonist, propanolol blocked the lipolytic effect on the muscle, suggesting the role of β-AR in lipolysis (Hagström-Toft et al., 1998). Langfort et al. (1999) demonstrated the stimulation of HSL by adrenaline in skeletal muscles, which indicates the alteration of skeletal muscle lipid metabolism, especially lipolysis by BA, as shown previously in Figure 2.11. However, the effect of BA, particularly in the molecular level, in the lipid metabolism of the skeletal muscle is not clear and still needs to be elucidated.

2.5.5 Effects of BA in adipose tissue

2.5.5.1 Induced lipolysis and inhibit lipogenesis

Direct stimulation of BA on adipose tissue, mediated via β-AR often related with its ability to enhance lipolysis and reduce lipogenesis. BA enhances the mobilization of lipid from adipose tissue, as well as attenuates the synthesis of fat, which might lead to the reduced hypertrophy of adipose tissue and eventually reducing carcass fat mass in animals (Mersmann, 2002). The indicator of lipolysis, the elevation of NEFAs and glycerol, have been observed in the

adipose tissue explants from pigs treated with ractopamine (Peterla and Scanes, 1990). Liu and Mills. (1990) also demonstrated that ractopamine and clenbuterol inhibit the lipogenesis, by counteracting the insulin action (stimulator for lipogenesis), probably via reducing insulin sensitivity in swine adipocytes *in vitro*. The inhibition of malic enzyme, which is a lipogenic enzyme, was also reported by the same research group (Mills et al., 1990). In addition, the inhibition of the expression of factors such as fatty acid synthase (FAS), sterol regulatory element binding protein-1 (SREBP-1), and glucose transporter-4 (GLUT-4), which are associated with lipogenesis has been observed, in adipose tissue of ractopamine treated finishing pigs (Halsey et al., 2011; Reiter et al., 2007).

The BA have been shown to induced lipolysis and inhibit lipogenesis in rat adipocytes, as well as inhibit preadipocyte growth *in vitro* (Yang and McElligott, 1989). The stimulation of lipolysis in white adipose tissue of rat treated with non-selective BA, isoprenaline, was predominantly modulated via β_1-AR (Louis et al., 2000). The increase in glycerol release and decrease the amount of ^{14}C-acetate incorporate into fatty acid (indicator for lipogenesis rate) from rat adipose tissue *in vitro* has been observed by dose-dependent stimulation of selective BA, isoproterenol (Duquette and Muir, 1985; Duquette and Muir, 1984; Del Boca and Flatt, 1969). The *in vitro* test by comparing different types of BAs demonstrated that the reduction in fat deposition was stimulating by inhibition of lipogenesis rather that enhancing lipolysis (Duquette and Muir, 1985). The effect of BA in inducing lipolysis have been demonstrated to be mediated by HSL, as shown in Figure 2.11,

In ruminants, intravenous isoproterenol infusion induced lipolysis in lambs and heifers (Johnson et al., 2014). Cimaterol and clenbuterol treatment in lambs significantly induced elevation of plasma NEFAs, suggesting enhancement of lipid mobilization and stimulated lipolysis in the adipocytes *in vitro* (Smith, 1987). In bovine, BA treatment with agents such as L-644,969, induced reduction in adipose tissue mass, as demonstrated by Chwalibog et al. (1996).

A study by Page et al. (2004) ractopamine administration induced apoptosis in mice adipocytes, which could be due to the very high dose used (800ppm) (Ferreira et al., 2013). Doses which were closer to those used commercially, up to 20ppm, apparently did not induced lipolytic effect in pigs, or the effect was temporary (Dunshea and King, 1995). In some cases, acute BA treatment induced elevation of plasma NEFAs level in swine and in cattle, however the response is reduced with chronic administration of BA in cattle and in sheep (Mersmann, 1998). In addition, there was also some discrepancies between *in vivo* and *in vitro* findings. Example, in pigs treated *in vivo* with clenbuterol demonstrated an increased in NEFAs, while the lipolytic activity in adipocyte *in vitro* is unaffected, suggesting that the *in vivo* reduction in fat deposition and lipolytic activity does not present as modulation of fatty acid metabolism *in vitro* (Mersmann, 2002). The duration of treatment also influenced the findings. In sheep, chronic clenbuterol treatment stimulated lipid mobilization by reduced carcass fat and increase NEFAs level, however lipogenic activity was increased in subcutaneous fat. While in cattle, although chronic clenbuterol feeding reduced the fat deposition in all sites, the lipogenic enzyme activities only reduced in subcutaneous fat, but not in intramuscular and perineal fat (Yang and McElligott, 1989).

2.5.6 Effects of BA in liver

The release of adrenaline from the adrenal medullar, and subsequent stimulation of the β-AR, stimulates the release of glucagon. Subsequently this stimulates glycogenolysis (in fed states), and acceleration of gluconeogenesis (in starved states) thereby enabling elevated hepatic glucose production (Philipson, 2002; Nonogaki, 2000). During the activation of β-AR, stimulation of adenylylate cyclase and subsequently cAMP-dependent pathway, activates glycogen phosphorylase, which catalyse glycogenolysis reaction (Nonogaki, 2000). This effects also have been shown using transgenic F28 mice, which displayed β_2-AR overexpression on their hepatocytes. The clenbuterol treatment on F28 mice was reported to affect the expression of gluconeogenic and glycolytic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase, and L-pyruvate kinase, as well as reduced hepatic glycogen and blood glucose levels,

and antagonized by propranolol (Erraji-Benchekroun et al., 2005). However, the *in vivo* rates of fatty acid synthesis in the liver of the cimaterol-treated rats were unaffected, probably due to suppressed conversion of carbohydrate into fatty acids or reduction of adipose tissue gain and induction of skeletal muscle gain, which might contribute to the suppression on fat synthetic capacity by cimaterol (Eadara et al., 1989).

As a summary, the effect of BA is appeared to be strong in protein metabolism, which involved in the muscle hypertrophy rather than lipid metabolism. However, BA is also involved in the catabolic activity of the lipid, promoting either reduced lipogenesis or induced lipolysis, but not as strongly as GH.

2.6 GENERAL SUMMARY

The increase in global population is expected to the increase in food demands, particularly for animal protein such as meat. In response to this challenge, the metabolic modifiers were developed, and are proven to increase production, improve feed efficiency and animal growth rates, as well as improve economic benefits. These metabolic modifiers, such as BA and GH, alter various mechanisms in protein and lipid metabolism and turnover, which ultimately induced muscle hypertrophy, while decrease fat deposition. This metabolic action of BA and GH appear to induce nutrient repartitioning, among adipose tissue and peripheral tissues. However, the metabolic changes of BA and GH, whether directly or indirectly mediated by IGF-1, on the transcriptional level in the target tissues are still unclear. Although BA and GH have been demonstrated in affecting lipid metabolism, it is not yet clear what level of crosstalk there is, and what the relationship is, between the effect of BA and GH on metabolic active tissues (skeletal muscle, adipose tissue and liver) involved in lipid metabolism, with respect to lipogenesis and lipolysis at the molecular level. Thus, in this thesis, the effects of BA and GH, as well as the effects of dibutyryl cyclic adenosine monophosphate (dbcAMP) and des (1-3) IGF-1, on the regulation of the target genes involved in those processes were determined, both *in vivo* and *in vitro*, respectively, and the possible mechanism altered by both

agents were identified, which may benefit in improving the sustainability in animal production.

2.6.1 Objectives

The objectives of this study are:

- To carry out development to establish a method for:
    - RNA extraction from the subcutaneous adipose tissue for the determination of expression of specific mRNAs
    - Determining the media conditions required to encourage the depositions of lipid droplets in differentiating C2C12 cells in culture.

- To carry out development to establish a method for the transdifferentiation of muscle cells into adipocytes, so that it could be used as a model of "true" muscle by consisting of myogenic (myotube formation) and adipogenic (lipid droplets formation indicating the intramuscular fat) properties.

- To examine the effect of BA and GH on the regulation of lipogenic and lipolytic gene expression in skeletal muscles, adipose tissue and liver and relate this to the response of lambs to BA and GH, particularly the effects on carcass characteristics, as well as tissue lipid profiles, and circulating metabolite concentrations.

- To determine if the effects of BA and GH *in vivo* (sheep) is similar to the changes seen *in vitro* in C2C12 myoblast cell lines when they are treated with the agents dbcAMP and Des (1-3) IGF-1 which will affect the signalling pathways stimulated by BA and GH *in vivo.*

2.6.2 Hypothesis

The hypotheses of this study are:

- Optimizing the methods in the RNA extraction, particularly from the subcutaneous adipose tissue, will result in higher RNA concentration yields.

- The volume used in changing the media for cells in culture will increase the development of the lipid droplets in the cells.

- By incubating the C2C12 myogenic cell line in adipogenic media, these cells will undergo transdifferentiation into adipocytes, thereby exhibit the characteristics of "true" muscle by containing both myocytes and adipocytes

- The metabolic modifiers, BA and GH affect lipid deposition, by either stimulating lipolysis or inhibiting lipogenesis or both. The metabolic changes, particularly in the gene transcription level in the skeletal muscles, adipose tissue and liver correspond to the effectiveness of both metabolic modifiers in bring about these changes.

- The effect of dbcAMP and Des (1-3) IGF-1, which mimic the action of BA and GH, *in vitro* (on the transdifferentiated-myoblast cell lines in the adipogenic media) will activate pathways and cause effects similar to those seen in *in vivo* (sheep).

3. MATERIALS AND METHODS

3.1 MATERIALS

All chemicals were analytical grade and purchase from Sigma-Aldrich Ltd (Poole, UK) or Fisher Scientific Ltd (Loughborough, UK). The kits used for various molecular biology techniques were purchased from the companies as specified in the text. All quantitative PCR was carried out using a kit from Roche Ltd (Burgess Hill, UK) or Invitrogen (Paisley, UK). All cell culture experiments were carried out using the Division of Nutritional Sciences cell culture facilities.

3.2 ANIMAL TRIALS

3.2.1 Sheep trial

Animal procedures were conducted in accordance with the provisions the UK Home Office Animals (Scientific Procedures) Act of 1986. This trial was carried out by Dr Krystal Hemmings and Dr Zoe Daniel. All the data on animal growth characteristics were analysed independently as part of this thesis.

3.2.2 Animal sampling

Male twin lambs (Mule × Charolais) were kept with their mothers until 53 ± 4 days of age, when they were weaned and pairs were categorized into two age groups, day 60 (D60) and day 120 (D120) groups. Only the D120 lambs were used for the experiment described in this thesis. Creep feed (48.48% Barley, 27% Soya, 15% nutritionally improved straw, 5% Molassed feed meal, 2.5% Vitamins and minerals, 1% Limestone, 0.5% Salt, 0.5% Vegetable oil and 0.02% Minsal g109 E50) was given about 28 days before weaning and *ad-libitum* access from weaning onwards. Before treatment, at day 120, lambs were confined individually with free access of feed. The lambs, which were not significantly different in starting body weight, were randomly categorized into

three treatment groups. The control group (C, n=11), the GH group (n=10) were treated with bovine GH formulation (POSILAC, Monsanto), at a dose of 3.75 mg/kg body weight, injected subcutaneously, on the nape of the neck as described by McLaughlin et al. (1994), and BA group (n=10), which diet containing cimaterol (Pfizer Ltd, UK), at 10 ppm (10 mg/kg). All groups were fed *ad-libitum* and no food restriction prior to slaughter. After 6 days of treatment, all lambs were slaughtered utilising the commercial practice procedure in the School of Biosciences, University of Nottingham abattoir and the whole blood samples were into collected into EDTA tubes, centrifuged at 3000 g for 10 minutes at 4°C for the collection of plasma. Both whole blood and plasma were stored at -40°C. The muscles, *longissimus dorsi* (LD) and *supraspinatus* (SS), *semitendinosus* (ST), *vastus intermedius* (VIN), *vastus lateralis* (VLAT) muscles, liver, and subcutaneous adipose tissue were collected, weighted, followed by snap frozen with liquid nitrogen, and stored at -80°C for further analysis.

3.2.3 Lipid extraction and fatty acid methyl ester profile

3.2.3.1 Lipid extraction from tissues

300-350 mg of tissues (*suprasupinatus* muscle, subcutaneous adipose tissue) were homogenized using a Polytron tissue homogenizer (Kinematica AG, Lucerne, Switzerland) in 1.6 ml sodium sulphate (1 g in 15 ml water) and mixed with 5.4 ml of hexane: isopropanol (3:2, v/v) and 2 ml sodium sulphate (1 g in 15 ml water). The samples were then vortexed for 30 seconds followed by centrifugation at 2000 rpm for 15 minutes at room temperature. After centrifugation, the top solvent layer was transferred into a fresh tube and dried down under gas nitrogen. The dried extract was re-suspended in 1 ml hexane and stored at -20°C until further used.

3.2.3.2 Separation of lipids by thin layer chromatography (TLC)

The TLC solvent system was prepared by the hexane, diethyl ether and glacial acetic acid mixture at 90:30:1 (v/v) ratio. 121 ml of the mixture was poured into

the TLC tank and the lid was affixed and left for at least 30 minutes to evaporate and saturate the tank atmosphere. Meanwhile, 100 µl of sample (from above experiment) was applied onto the TLC plastic-sheets-silica gel-60 (20 cm x 20 cm). Each sample was spotted along a 1.5 cm line, which was 1.5 cm above from the bottom of the sheet, and dried. The sheet was then transferred into the tank-containing TLC solvent, with the lid weighed down on top, to ensure the atmosphere inside the TLC system was maintained, and the plate was positioned with the sample loading spots at the bottom so the bottom of the plate was inserted into the solvent. The diagram for TLC sample spots and separation were shown in the Figure 3.1. The solvent moved upward along the plate from the position of sample spots carrying the sample up sheet and separating its constituents. When the solvent reached approximately 1.5 cm from the top of the sheet, the sheet was taken out and was then transferred into the iodine tank to visualize the sample separation by iodine vapour. The sheet was left in the open air for de-staining overnight. The stained bands on the sheet were marked, and will be used for the next extraction.

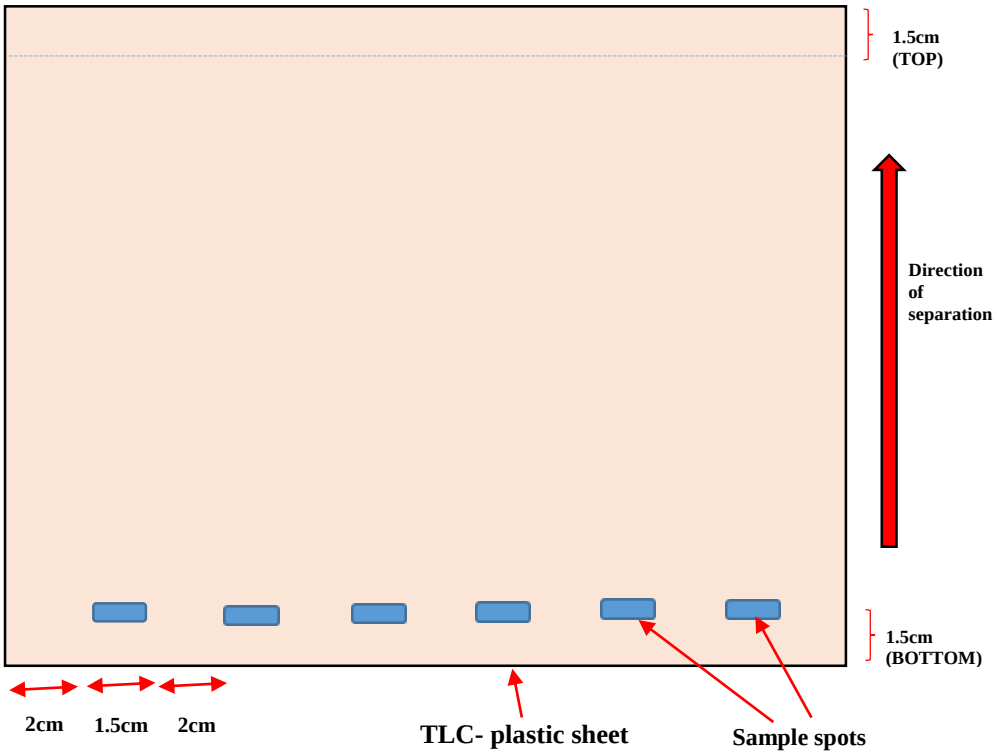


Figure 3.1: Diagram for sample spot on the TLC-plastic sheet. Each sample was spotted along a 1.5 cm line, which was 1.5 cm above from the bottom of the sheet, with 2.0 cm between sample spots. The sheet was taken out once the separation reached 1.5 cm from the top of the sheet.

3.2.3.3 Extraction of triglyceride and cholesterol esters from the TLC plates

The target band produced from the separation on the TLC plates were cut and transferred into vials containing 2 ml of hexane: isopropanol (3:2, v/v). The sample/solvent mixture were leaved for 30 minutes with frequent mixing to elute the lipid from the silica. The mixture was then transfer into the LP4 tubes using glass pipettes, and spun at 3000 rpm at 25°C for 10 minutes to remove the silica precipitate the carried over from the previous step. The mixture was transferred into fresh, weighed LP4 tubes, and dried under nitrogen. The dried tubes were re-weighed to get an estimation of extracted lipid. The wall of the tubes was rinsed with 1 ml of hexane, and vortexed to re-suspend the sample. The sample were then stored frozen at -20°C before further analysis.

3.2.3.4 Synthesis of fatty acid methyl ester (FAME)

The sample (from the previous experiment) was vortexed (if frozen) and transferred into the screw-capped methylation tube, followed by dried under nitrogen gas. 700 µl of 10 M potassium hydroxide and 5.3 ml of methanol were added into the sample and capped. The sample was then incubated in a water bath at 55°C for 90 minutes, with 5 seconds vortexed to mix the sample for every 20 minutes. The sample was cooled down in cold water for 10 minutes. After that, 0.58 ml of 12 M sulphuric acid was added into the sample, followed by incubated and mixed as before. After cooled down, the sample was added with 3 ml of hexane and vortexed for 30 seconds. The sample was then centrifuged for 5 minutes at 2000 rpm at room temperature. After centrifuged, the top layer was transferred into the new LP4 tube and stored at -30°C until analysis with gas chromatography.

3.2.3.5 Gas chromatography

Quantitative determinations of FAMEs were conducted using a Clarus 500 gas chromatograph (Perkin Elmer, Connecticut, USA) with a flame ionization detector (FID) and capillary column CP-Sil 88 (100 m × 250 µm) (Varian Inc., CA, USA) with a stationary phase (100% cyanopropyl polysiloxane) with the

thickness of 0.2 μm. The injection volume was 1.0 μL, the temperature of injection port was 250 °C with the split ratio of 1:50 (for the muscle tissues) and 1:100 (for the subcutaneous adipose tissue). Hydrogen was used as a carrier gas. The temperature program was initially set and held at 45°C for 4 min, and ramped up to 175°C at 13°C per min, and held for 27 min, followed by second ramped up to 215°C at 4°C per min, and held for 35 min, until the end of the cycle. Identification of FAMEs was performed by comparing their retention times with those of reference standards (Supelco^{TM} 37-Component FAME Mix). The results of FAs were demonstrated as percentages of total FAMEs.

3.2.4 RNA extraction from tissues

LD, SS muscles, liver and subcutaneous adipose tissue were crushed in liquid nitrogen using a pestle and mortar. 100 mg of samples were homogenised on ice with 1 ml of Trizol (Invitrogen, Paisley, UK) with Polytron tissue homogenizer (Kinematica AG, Lucerne, Switzerland). The homogenized samples were then transferred to a fresh 1.5 ml tube. Meanwhile, the homogenizer blade was rinsed and cleaned with distilled water and dried or wiped between each sample to prevent any cross-contamination. Homogenates were centrifuged at 12000xg (Microfuge 22R centrifuge, Beckman Coulter Inc.) at 2-8°C for 10 minutes. The supernatant transferred into a fresh 1.5 ml tube. 0.2 ml of chloroform was added into the supernatant and shaken vigorously for 15 seconds, followed by incubation at 15-30°C for 3 minutes. The mixture was then centrifuged at 12000xg for 15 minutes at 2-8°C and the upper colourless aqueous phase was transferred to a fresh 1.5 ml tube, mixed with 0.5 ml isopropanol for liver and subcutaneous adipose tissue samples, or 0.25 ml of isopropanol and 0.25 ml of high salt precipitation buffer (0.8 M sodium citrate/1.2 M NaCl) for LD and SS samples, for the glycogen removal from RNA, followed by incubation at 15-30°C for 10 minutes. The precipitate was then centrifuged as above. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% (v/v) ethanol, vortexed and recentrifuged at 7500xg for 5 minutes at 2-8°C. The supernatant (ethanol phase) was removed and the pellet was air dried for 5-7 minutes. The pellet was then re-suspending in 40 μl of RNase free water and stored at -80°C.

The additional step of DNase digestion was performed to remove any contaminating genomic DNA (gDNA) in liver, LD and SS muscles. However, this step was not applied to the subcutaneous adipose tissue samples as the reduced amount and degradation of total RNA upon DNase step (as described in Section 3.5). 5 µl of DNase 10x buffer and 5 µl DNase (1000 units) (Promega Corporation, USA), were added into 40 µl of total RNA (from previous step), mixed and incubated at 37ºC for 30 minutes. The denaturation process was stopped by adding the 150 µl of RNase free water and 200 µl of phenol/chloroform/isoamylalcohol solution (25:24:1) saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma-Aldrich Co. USA), followed by vortexed and centrifuged at 13000g for 5 minutes at 2-8°C. The upper layer containing RNA extracts were transferred carefully into fresh tube, and precipitated with 15 µl of 3M sodium acetate pH 5.5 (NaAc) and 375 µl of 100% (v/v) ethanol at -80°C for 1-2 hours or longer. After precipitation, the precipitate was centrifuged at 13000xg for 10 minutes at 2-8°C. The supernatant was discarded and the RNA pellet was washed with 1ml of 75% (v/v) ethanol, vortexed and re-centrifuged as above. The ethanol was removed and the pellet was air dried as before. The RNA was then re-suspended in 40 µl of RNase free water, and stored at -80°C for further analysis.

3.2.5 Determination of RNA concentration

The concentration and purity of RNA that was diluted in nuclease free water were measured using spectrophotometry (Nanodrop ND-1000, Thermo Scientific, Wilmington, USA). Ratio of absorbance at wavelengths 260/280 and 260/230 nm were used to assess the purity of nucleic acids. Ratio reading of 1.8-2.0 at 260/280 was accepted for RNA. For all samples, the concentration of RNA was normalised to 100ng/µl (except RNA from subcutaneous adipose tissue samples were normalized at 50ng/µl) for subsequent use in gel electrophoresis or cDNA synthesis.

3.2.6 Running agarose gel electrophoresis for RNA integrity

To cast the gel, the edges of gel cassette were taped with autoclave tape to prevent leaking of the molten gel during gel casting, and a comb was inserted in the apparatus, for the template of the gel loading slots. The 1% (w/v) agarose gel was prepared by heating 0.5 g agarose in 50 ml of 1X TAE buffer (40 Mm Tris, 1 Mm EDTA, 0.12% (v/v) acetic acid). Then, the agarose was heated in microwave, reached the boiling point until it completely dissolved and became clear. The agarose was left to cool down to approximately 50°C, and poured into a gel cassette. The gel was then transferred into the electrophoresis tank apparatus and filled with 1X TAE buffer. 5 µl of total RNA samples (which already normalized at the same concentration, as described above) were mixed with 5 µl of RNase-free water and 2 µl of loading dye (for 6X loading buffer, 50% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 5 mM sodium phosphate pH 6.8). The whole loading mixture RNA was then pipetted into agarose gel loading slots carefully, and ran at 100 V for 45-60 minutes. The gel was then transferred into 1µg/ml ethidium bromide for 20-30 minutes for nucleic acids staining, and then soaked in water for de-staining. The gel image was captured using Gel Doc system and MultiAnalyst (Bio-Rad, UK). The observation of the formation of two distinct bands ribosomal RNA (rRNA) bands (18S and 28S), indicating the good quality and intact of RNA (Figure 3.2), which can be proceed to cDNA synthesis.

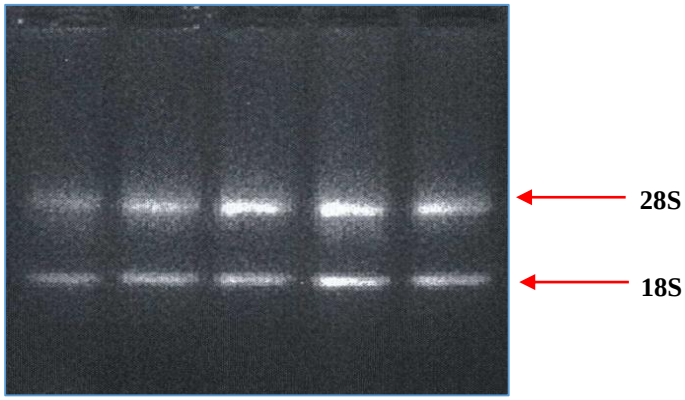


Figure 3.2: The typical rRNA bands on the agarose gel after stained with ethidium bromide. The presence of two bands corresponding to 28S (upper band) and 18S (lower band) rRNA, indicating good quality RNA.

3.2.7 cDNA synthesis

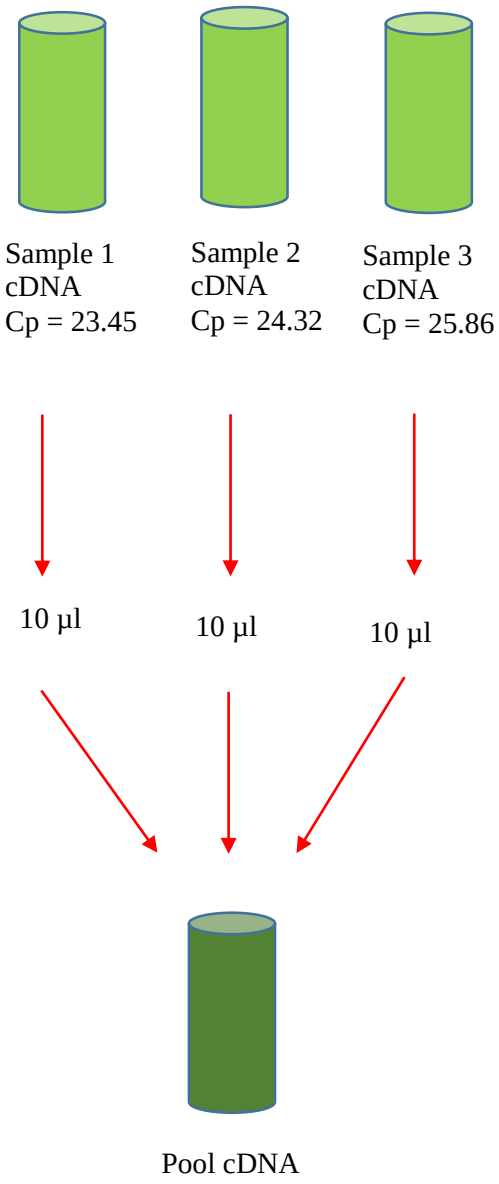
Total RNA was diluted to 100ng/µl (for LD muscle, SS muscle and liver samples) and 50ng/µl (for subcutaneous adipose tissue samples) on a sterile, nuclease-free, thin-walled PCR tubes/plates; the reverse transcription reaction was carried out to synthesise first strand DNA using a cDNA synthesis kit (Transcriptor First Strand cDNA Synthesis Kit, Roche, Burgess Hill, UK) according to the manufacturer's protocol. In the PCR tubes/plates, 5 µl of diluted RNA (as above) was mixed with 2 µl of Random Hexamer primer (60 µM) and an appropriate volume of DNase RNase free water (Sigma-Aldrich Ltd, Poole, UK) to a final volume of 13 µl. For large number of samples PCR plates were used. If plates were used, lids, which were attached together in strips that allowed the wells in a column to be sealed, were placed the plate. To ensure mixing of the reaction mixture, the tubes/plates were then shook and spun briefly in mini spinner centrifuge (Labnet International Inc, USA). The mixture was incubated at 65°C for 10 minutes on a thermal block cycler of PCR machine to denature RNA secondary structures. After the incubation, the mixture was immediately cooled on ice. On ice, the annealed primer mix was added 4 µl of 5x concentrated Transcriptor Reverse Transcriptase Reaction buffer, 0.5 µl of protector RNase inhibitor (final concentration 20 U), 2 µl of deoxynucleotide mix (10 mM each), and 0.5 µl of Transcriptor Reverse Transcriptase (final concentration of 10 U) to a final volume of 20 µl. The plate was placed in the thermal cycler PCR machine and incubated for 10 minutes at 25°C followed by 30 minutes at 55°C. The Transcriptor Reverse Transcriptase was inactivated at subsequent incubation at 85°C for 5 minutes. The mixture was then transferred on ice to stop the reaction. The mixture was then diluted with 80 µl of RNase-free water to create a 100 µl cDNA stock solution. The stock cDNA was stored at -20°C until used for real-time quantitative PCR (RT-qPCR) analysis.

3.2.8 Real–time qPCR

3.2.8.1 Preparation of pool cDNA

All cDNA synthesized (from above), were subjected into the Real-Time Quantitative Polymerase Chain Reaction (qPCR). 5 µl of cDNA was mixed with PCR reaction mix (Mixture of 7.5 µl of 1X SYBR Green Master Mix (Roche, Burgess Hill, UK), 0.45 µl of Forward and Reverse Primers (10µM; final concentration of 0.3µM each), and 1.6 µl of DNase RNase-free water (Sigma-Aldrich Ltd, Poole, UK) (dependent of the number of samples) with the final volume of 15 µl per well in 384-well plate. The plate was covered with adhesive sealing foil, to avoid evaporation during heating the PCR machine, and spun briefly with mini spinner centrifuge (Labnet International Inc, USA), to ensure complete mixing. The plate was then transferred into the Lightcycler 480 (LC-480) (Roche, UK) using the following conditions; 1 cycle of pre-incubation (95°C for 5 minutes), followed by 40 cycles of amplification at (95°C for 10 seconds, down to 60°C for 15 seconds, and up to single acquisition mode at 72°C for 15 seconds), then 1 cycle of melting curve (95°C for 5 seconds, down to 65°C at 1 minute, and up to continuous acquisition mode at 97°C), before 1 cycle of cooling at 40°C for 10 seconds.

The pool cDNA was prepared according to their individual crossing point (Cp value). The average and standard deviation of the crossing point of whole cDNAs were calculated (average ± (2 x standard deviation) and individual cDNA which had crossing point within the range were considered to make a pool and were mixed together in one tube (Figure 3.3). This pool was used to produce standard curve to check the efficiency and linearity of the PCR reaction (Ryan et al., 2013). The example is shown and described as follow (Figure 3.3):



1. First, the average of the whole Cp was calculated:
= [(Cp cDNA 1) + (Cp cDNA 2) + (Cp cDNA 3)]/3
= [(23.45) + (24.32) + (25.86)]/3
= **24.54**

2. Then, the standard deviation was calculated:
= $[(23.45-24.54)^2 + (24.32-24.54)^2 + (25.86-24.54)^2]/3$
= 1.22
If 2 x standard deviation
= 2 x 1.22
= **2.44**

If average ± (2 x standard deviation)
= 24.54 ± 2.44
= **22.1 and 26.98**

3. Thus all cDNA with Cp value between 22.1 and 26.98 were considered for the pool preparation, which were cDNA from sample 1, 2, and 3. 10 µl of cDNA from each sample were transfer into new tube (Pool cDNA).

Figure 3.3: Preparation of the pool cDNA. The Cp value from each cDNA, (obtained from the RT-qPCR reaction), was used for the calculation of [average ± (2 x standard deviation]. 10 µl from individual cDNA were transferred into new tube (pool cDNA), which can be stored frozen at -20°C, until used.

3.2.8.2 Preparation of the standard curve

An aliquot of the cDNA pool (from above) was serially diluted (two-fold serial dilutions e.g: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128) of the original pool. 5µl of original pool and all serial diluted pool were mix with PCR reaction mix (as described above), on the different well but on the same 384-well plate, and

transferred into the LC-480 (Roche, UK) (with the same protocol described above) for the production of standard curve. The linear standard curve was produced with optimal gradient at -3.3 and optimal efficiency at 2.0, which indicated good reaction (Figure 3.4). The melting curve was also generated to check if any contamination or primer-dimer formation occur in the reaction. If the reaction was good, the melting curve only produce one product (one amplicon, indicated by producing one high curve) (Figure 3.4).

3.2.8.3 Assessing of the gene expression from the samples

The first strand cDNA for individual samples that were to be assessed for gene expression were further diluted by taking the individual stock and diluting each separately to 1:8. On the same plate, the sample (diluted to 1:8) and standard curve, were mixed with PCR reaction mix (as described above) and were then run in the LC-480 (Roche, UK) (with the same protocol described above).

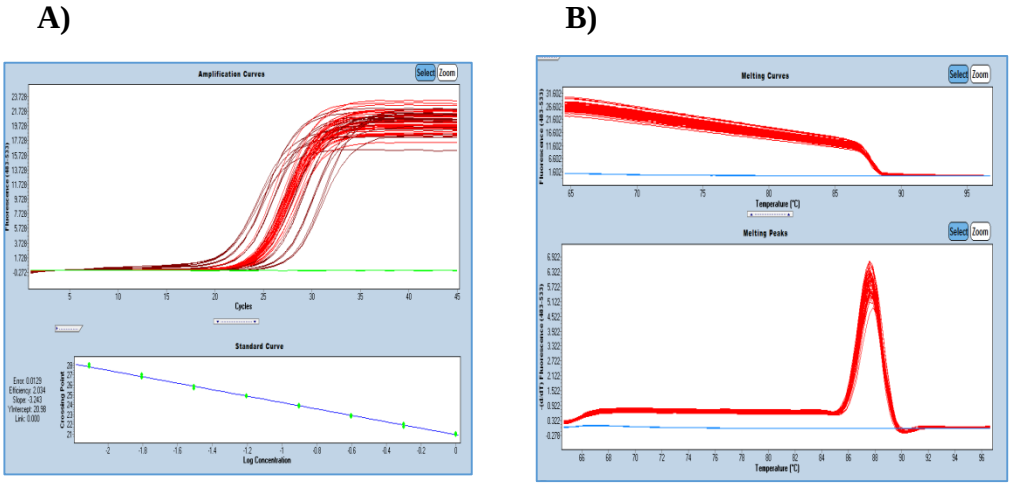


Figure 3.4: Output images from the LC-480. A) The amplification curve (upper), with the standard curve (lower) generated from the Lightcycler 480 software (version 1.5.1.62). The standard curve generated by the serial dilution of the pool cDNA, with linear slope, gradient at -3.2 (optimal gradient -3.3), and efficiency at 2.0 (optimal efficiency is 2.0). The individual cDNA sample dilution (1:8), were fallen within the range of the standard curve, and the gene expression of each cDNA were calibrated using the standard curve and calculated in LOG concentration unit. B) The melting curve images, which showed only one high curve, indicated the reaction only producing one product (one amplicon).

3.2.8.4 Oligreen for determining cDNA quantity

The alternative to the use of a reference gene to normalise gene expression is through use of oligreen (Invitrogen, Paisley, UK). Identification of a reference gene can sometimes be problematic as experimental interventions can often cause significant shifts in global gene expression which makes it difficult to identify a gene whose expression does not change due to treatment. The oligreen protocol utilises a fluorescent chemical that specifically binds with single stranded DNA molecules (Rhinn et al., 2008), therefore it is possible to normalise the gene expression to total first strand cDNA concentrations.

In all the gene quantification procedures described in this thesis the concentration of extracted total RNA was assessed after DNase treatment (except the subcutaneous fat sample), then normalised to the same concentration which was then followed by the samples being assessed by gel electrophoresis for quality as well as quantity before first strand cDNA was generated.

A working solution of oligreen sDNA solution was made by diluting 1:200 in 1x TE buffer (e.g. 5µl oligreen into 1ml 1x TE buffer). A standard curve was made by a serial dilution standard curve from a pool of cDNA. To 5µl of each aliquot of the cDNA standard curve was added 5µl of working oligreen reagent, likewise for 5µl of each sample's first strand cDNA on a 384-well plate. The 384 plate was spun the then incubated in dark for 5 min at room temperature and run on Roche LC-480 (Roche, UK), consisting of one cycle of continuous acquisition mode which was set to read at 95°C. The melting curve against the fluorescence was generated, and were converted into numerical data using Microsoft Office Excel 2013 (Microsoft Inc., USA). The standard curve was generated from the data, and thereby determine the relative quantity of cDNA for each sample.

3.2.8.5 Primer design and bioinformatics

The desired genes, comprised of forward and reverse primers, which to be assessed by the real-time qPCR, were designed from the specific sequence of the gene of interest. The desired genes were searched via NCBI (National Centre for

Biotechnology Information, https://www.ncbi.nlm.nih.gov/) database. Due to the lack of verified sequences for sheep (ovine), the desired gene transcripts were identified via Expression Sequence Tag (EST) cDNAs or partial length cDNAs. For target transcripts where there were no available sheep sequences encoding the same protein, but were available in different species, these were retrieved from the database. Alignment of the conserved sequences between species of a multiple sequence of mRNA/cDNAs was carried out by Clustal W2 to identify the areas of conserved similarity across species. This allowed the generation of composite cDNA sequences where there was the greatest similarity between species. These sequences were used for primer design. The FASTA sequence of the target genes was subjected into the primer designing tool, the Primer Express (Applied Biosystems, UK), and compared with the genomic sequences provided by EnsEMBL, for the identification of exon-intron junctions/boundaries, which used Taqman Quantification as default parameter. Outputs from the software were the location in the sequence, and secondary structure information as well as primers and probe identified sequences and their lengths. The conserved sequence of the primers was then subjected to the standard nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), in order to ensure the primers were only specific to the desired genes.

Sheep and mouse primers used in this study are shown in Appendix 7.1. Primers were manufactured by Sigma (UK), in a dry form. Upon arrival, the primers were re-suspended to 100mM with RNAse free-water (according to the manufacturer information attached with the primers), and then diluted 1:10 with RNAse-free water. The primers were then tested with Real-time PCR to ensure only one amplicon/product was being produced in the PCR reaction (as shown in Figure 3.4(B)), indicating that the primers were working.

3.2.9 Metabolomics analysis

This metabolomics profile analysis was carried out by Metabolon Inc., Durham (NC 27713. www.metabolon.com). As describe above, the plasma samples were collected from all lambs, from each treatment; C (n=11), BA (n=10), and GH (n=10). The samples were then stored frozen and sent to Metabolon, for metabolomics analysis.

3.2.9.1 Sample preparation

The sample preparation process was carried out using the automated MicroLab STAR® system (Hamilton Company, USA) according to Metabolon's standard protocol, which is designed to remove the protein fraction while allowing maximum recovery of small molecules or metabolites. An aliquot from the plasma sample was taken and pooled for the creation of "Client Matrix" (CMTRX) samples. These CMTRX samples were injected throughout the platform run and served as technical replicates (Figure 3.5), allowing variability in the quantitation of all consistently detected biochemicals to be determined and overall process variability and platform performance to be monitored. The resulting extract was divided into two fractions; one for analysis by LC and one for analysis by GC. Samples were placed briefly on a TurboVap® (Zymark, CA, USA) to evaporate the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS.

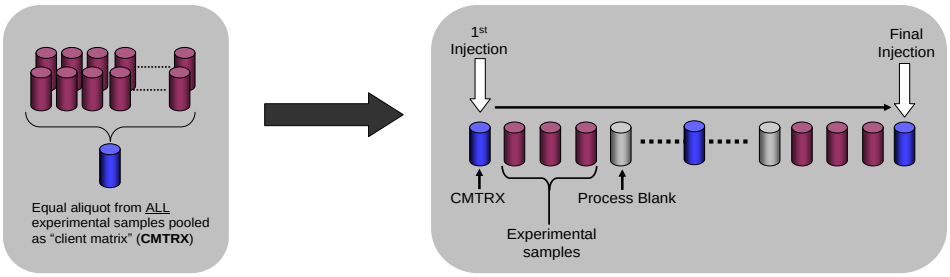


Figure 3.5: Preparation of client-specific technical replicates. A small aliquot of each client sample (purple-coloured cylinders) is pooled to create a CMTRX technical replicate sample (blue-colored cylinder), which is then injected periodically throughout the platform run. Variability among consistently detected biochemicals can be used to calculate an estimate of overall process and platform variability

3.2.9.2 Data collection and normalization

The CMTRX technical replicate samples were treated independently throughout the process as if they were client study samples. All process samples (CMTRX, GROBs – a mixture of organic components used to assess GC column performance, process blanks, etc.) were spaced evenly among the injections for each day and all client samples were randomly distributed throughout each day's run. Data were collected over multiple platform run days and thus, 'block normalized' by calculating the median values for each run-day block for each individual compound. This minimizes any inter-day instrument gain or drift, but does not interfere with intra-day sample variability. Missing values (if any) were assumed to be below the level of detection for that biochemical with the instrumentation used and were imputed with the observed minimum for that particular biochemical.

Table 3.1 showed the total number of metabolites detected in this study. This total was corresponded to many biochemicals that matched a named structure in our reference library (named). The remaining were represented distinct chemical entities (which they might represented a single molecule of discrete molecular formula and structure), but they did not currently match a named biochemical in the reference library (unnamed/unknown).

Table 3.1: The number of biochemicals identified

Compound Classification	
Total	***405***
Named / Identified	277
Unnamed	128

3.2.9.3 Quality control

For QA/QC purposes, a number of additional samples were included with each day's analysis. Furthermore, a selection of QC compounds was added to every sample, including those under test. These compounds were carefully chosen so as not to interfere with the measurement of the endogenous compounds.

Prior to the first step in the extraction process, the recovery standards were added for to each experimental and process standard sample to assess variability and verify performance of extraction and instrumentation, while the internal standards were added just prior to injection into the mass spectrometers, to assess variability and performance of instrument (Figure 3.6).

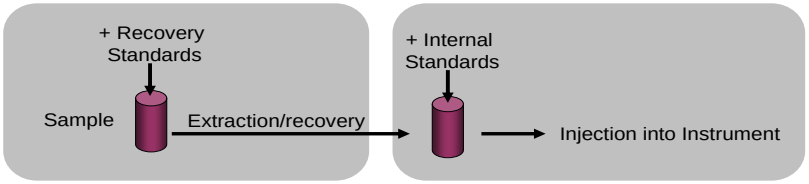


Figure 3.6: Multiple embedded QC standards in each sample. The recovery standards were added to assess variability of extraction and instrumentation, while the internal standards were added to assess variability and performance of instrument.

A measure of the platform variability was determined by calculating the median relative standard deviation (RSD) for these internal standards. Table 2 shows the median relative standard deviation (RSD) for the internal standards. Because these standards are added to the samples immediately prior to injection into the instrument, this value reflects instrument variation. In addition, the median relative standard deviation (RSD) for the biochemicals that were consistently measured in the CMTRX represents the total variability within the process for the actual experimental samples and the variability in quantitation of the endogenous metabolites within these samples (Table 2). Results for the CMTRX and internal standards indicated that the platform produced data that met process specifications.

Table 3.2. QC statistics

Quality Control Sample (Matrix)	Median RSD
Internal Standards	4%
Endogenous Biochemicals	9%

3.3 CELL CULTURE

3.3.1 Myoblast cell line growth

The adherent C2C12 skeletal myoblast cell line, derived from mouse, were grown in growth medium, Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, UK) and 1% (v/v) penicillin and streptomycin (P/S) (Sigma-Aldrich, UK). The cell line was maintained in a 5% CO_2 at 37°C. The grown cells were then passaged, and cultured in appropriate plates (six-well plates) until 70-80% confluence. The growth media then was replaced with differentiation medium, either myogenic or adipogenic differentiation media. The myogenic media contained DMEM containing 2% (v/v) horse serum (HS) and 1% (v/v) P/S. For adipogenic media, the cells were transferred into adipogenic induction media contained of DMEM containing 10% (v/v) hi-FBS, 1% (v/v) P/S, 0.5 mM isobutylmethylxanthine (IBMX), 1 µM dexamethasone (DEX), 850 nM insulin, 10 nM triiodothyronine (T_3) and 1 µM rosiglitazone (peroxisome proliferator activated receptor γ (PPARγ) agonist) media, indicated as day 0 of differentiation, and changed into another media with DMEM containing 10% (v/v) hi-FBS, antibiotics, 850 nM insulin, 10 nM T_3 and 1 mM rosiglitazone after 2 days in induction media. Both differentiation media were changed every two days for nutrient replenishment, until reached the end of the experiment.

3.3.2 Cell splitting and re-plating (sub-culturing)

When the cells reached confluent (~80% confluence), they started to differentiate, which will interfere with the development during the incubation with the differentiation medium. There is also a risk of cell death if the cells become over confluent. Thus, the splitting process is crucial to keep the cells in the proliferative state. Once the cells reached ~70% confluence, the culture medium was removed from the plate/flask, and the surface was washed with 10 ml of pre-warmed (37°C) phosphate buffer saline (PBS) to ensure all growth medium was removed. All PBS was pipetted out and was replaced with 2 ml of

pre-warm Trypsin-EDTA (Gibco, UK), with gently rocked to ensure trypsin contact with all cells, followed with 3-4 minutes incubation in 37°C incubator to detach all the cells from the plate/flask surface. The flask/plate was knocked to maximize cell detachment. The cells were observed under the microscope to ensure majority (>90%) cell detachment. After the cells had detached, 8 ml of growth medium (DMEM containing FBS and P/S as describe above) were added into the cell suspension, to inactivate the trypsin, and mixed by pipetting up and down several times. The cell suspension was then transferred into 20 ml universal (sterilin) tube, and centrifuged at 1000xg for 5 minutes at room temperature. After centrifuge, the supernatant was removed and the cell pellet was re-suspended in 10 ml growth medium. Approximately 1 ml of cell suspension was pipetted into the new T75 flask, followed by 14 ml of growth medium was added into the flask. The cells were then transferred into the incubator (5% of CO_2 at 37°C). The growth of the cells took approximately 2 days to reach sub-confluence before next re-splitting. However, regular observation was recommended to avoid over confluence of the cells.

3.3.3 Cell counting

The individual C2C12 cell in cell suspension was counted using a haemocytometer. The 70% ethanol was used to sterilize the haemocytometer and coverslip. The coverslip was moistened and fitted with the haemocytometer. Then, a small volume (10 µl) of cell suspension were transferred to one of the haemocytometer chamber, and were allowed to spread and fill the chamber by capillary action. The number of cells per ml of culture medium was calculated which correspondent to the total number of cells to be determined in the total volume of cell suspension media (Figure 3.7). The formula below can be used to determine the volume of cell suspension needed for seeding densities:

(Desired cell number/Total cell number) x Volume of cell suspension = Volume required

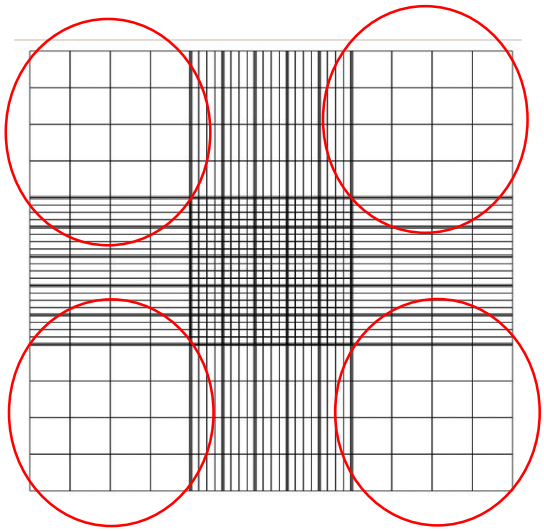


Figure 3.7: The grid in the haemocytometer. The number of cells, which were identified in 4 large squares, (red circled) were counted and the average was used to determine the number of cells per 10 µl of cell suspensions. The cells outside the large squares were not counted.

3.3.4 Freezing cells

The C2C12 cells were keep frozen in liquid nitrogen as a stock or long-term storage. Pelleted cells obtained from the sub-culturing step (as described above) were re-suspended in freezing medium (90% FBS and 10% dimethyl sulfoxide (DMSO)) in cryovials, and then transferred to a polystyrene container at -80°C for approximately 24 hours before being transferred to liquid nitrogen for permanent storage.

3.3.5 Cells harvest

The media was removed from the cells on the plate to be harvested for RNA. Then 200 µl of RNase-free phosphate buffered saline (PBS) was added to the cells. The cells were then scraped using the cell scrapper and collected directly into a sterile tube, snap frozen on dry ice and stored at -80°C until RNA extraction was carried out.

3.3.6 RNA extraction from cells

The total RNA was extracted from C2C12 cells using an RNA isolation kit (High Pure Isolation Kit for RNA isolation, Roche, UK) (which was different from the RNA extraction kit used in the animal trial), according to the manufacturer protocol. 400 µl of Lysis-Binding Buffer was added to the 200 µl RNase free PBS-containing cell suspension (from harvesting step) and vortexed for 15 seconds. The high pure filter tube from the kit was combined with the collection tube, and the whole sample was pipetted into the upper reservoir of the filter tube. The entire tube assembly was then centrifuged for 15 seconds at 8000xg at room temperature. After centrifuged, the filter tube was removed from the collection tube and the flow-through liquid in the collection tube was discarded. The filter tube and used collection tube were then re-combined.

Meanwhile, DNase mix (90 µl of DNase incubation buffer and 10 µl of 10KU lyophilized DNase I) was prepared and 100 µl of DNase mix was added onto the glass fibre fleece in the upper reservoir of the filter tube and incubated for 15 min at 15°C-25°C. After incubation, 500 µl of Wash Buffer I was added to the upper reservoir of the filter tube assembly followed by centrifuged for 15 seconds at 8000xg at room temperature. The flow-through was discarded. 500 µl of Wash Buffer II was then added to the upper reservoir of the filter tube assembly followed by a repeat centrifuged, and the flow-through was discarded. Another 200 µl of Wash Buffer II was then added and centrifuged for 2 minutes at 13000xg at room temperature to remove any residual wash buffer. The collection tube was discarded and the filter tube inserted into a sterile 1.5 ml micro centrifuge tube. 50-100 µl of elution buffer was added to the upper reservoir of the filter tube and centrifuged for 1 minute at 8000xg at room temperature to collect the eluted RNA. The RNA in the microfuge tube can be used directly for further analysis or stored at -80°C for long-term storage.

3.3.7 cDNA synthesis

Total RNA was diluted to 50ng/µl on a sterile, nuclease-free, thin-walled PCR tubes. The reverse transcription reaction was carried out to synthesise first strand

DNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, UK) (which was different from the cDNA synthesis kit used in the animal trial) according to the manufacturer protocol. In the PCR tubes and on ice, 250 ng of total RNA was mixed with 1 µl of Random Hexamer primer and 6 µl of DNase RNase free water, to a final volume of 12µl. Lids which were attached together in strips that allowed the wells in a column to be sealed were placed the plate. The plate was then shaken and spun in mini spinner centrifuge (Labnet International Inc, USA) to mix it completely. Then, the mixture was incubated at 65°C for 5 minutes on a thermal block cycler of PCR machine to denature RNA secondary structure. After the incubation, the mixture was chilled on ice immediately, followed by spun down to mix it, and replaced on ice. On ice, the annealed primer mix was added 4 µl of 5x Reaction Buffer, 1 µl of Ribolock RNAse Inhibitor (20U/µl), 2 µl of 10mM dNTP mix, and 1 µl of RevertAid RT (200U/µl) to a final volume of 20 µl. The sample was mixed and spun briefly. The plate was then re-transferred onto the thermal cycler on PCR machine and incubated for 5 minutes at 25°C followed by 60 minutes at 55°C. The reaction was terminated at subsequent incubation at 70°C for 5 minutes. The mixture was then transferred on ice to stop the reaction. The mixture was then diluted with 80 µl of RNase-free water to create a 100 µl cDNA stock solution. The stock cDNA was stored at -20°C for short term storage or for real-time quantitative PCR analysis or long-term storage at -70°C.

3.3.8 Oil Red-O and haematoxylin staining

Accumulation of lipid droplets was monitored by phase contrast microscopy. After 6 days of exposure to myogenic or adipogenic differentiation media, cells were stained with Oil Red-O to identify lipid droplets and counterstained with haematoxylin to delineate nuclear and myofibre structures. Briefly, media were removed and cells were fixed in 3.7% (v/v) formaldehyde at room temperature for 30 minutes. Cells were washed twice with pre-warmed (37°C) PBS and then with 60% (v/v) isopropanol. After washing, cells were stained with 0.3% (v/v) Oil Red-O (Sigma) for 30 minutes at room temperature. Cells were then washed once in 60% isopropanol and twice in tap water, before counterstaining with Harris haematoxylin (Sigma) for 3 minutes, all at room temperature. Excess stain

was removed by washing twice in distilled water and then another two times in Scott's tap water (0.24 M sodium bicarbonate and 0.03 M magnesium sulphate). Images were captured using an Olympus SZH10 microscope (Olympus, Southend-on-Sea, UK) and analysed using ImageJ Software (version 1.50I, National Institute of Health, USA) for the quantification of Oil Red-O staining. The ImageJ Software detected the Oil Red-O staining using a colour threshold and then calculated percentage area of staining for each image. Ten field of view images from each of three replicate wells were quantified for each treatment.

3.4 STATISTICAL ANALYSIS

For the animal (sheep) trials, the data for carcass characteristics, fatty acid profiles, metabolomics analysis, and gene expression analysis were analysed using SPSS 22.0 software (SPSS, Inc., IL, USA). The comparison between the control groups and the treatment groups (BA and GH) were analysed by Analysis of Variance (ANOVA), followed by Post-Hoc Dunnett's test. Meanwhile for the cell culture experiments, the data were analysed using Genstat for Windows 17th Edition (VSN International Ltd., UK) by ANOVA, followed by Bonferroni correction for any significance factors and interactions. All data were displayed as mean ± standard error of mean (SEM), and any $P<0.05$, were accepted as significance.

3.5 METHODS DEVELOPMENT AND OPTIMIZATION

3.5.1 Effect of DNAse treatment in RNA extraction from sheep subcutaneous adipose tissue sample

3.5.1.1 Introduction

Polymerase chain reaction (PCR), has been widely used for several molecular assessments such as gene mutation (Chamberlain et al., 1991) and tumour diagnosis (Bernard and Wittwer, 2002) by assessing the expression of the genes or protein activity. The ribonucleic acids (RNA) is a primary entity in PCR, as it involved in the protein synthesis pathway in the cells, which is transcribed by the deoxyribonucleic acid (DNA) and reflect the outcome of the PCR. Thus, the RNA extraction plays important roles in influencing the reliability and reproducibility of the PCR, which it depends on the good quality of RNA.

During RNA extraction, there are some conditions that should be followed to produce undegraded and high quality of RNA, which is free from any contamination such as genomic DNA (gDNA) contamination, which could potentially give amplification and gave false positive result during PCR analysis (Tavares et al., 2011; Pfaffl, 2005). Several techniques have been developed in order to reduce the risk of genomic DNA contamination in RNA, such as the use of DNase treatment (Norhazlin et al., 2015; Larman et al., 2011). However, the effect of DNase treatment on the RNA extracted from the subcutaneous fat has not yet been elucidated. In this methods optimization, we found that the RNA extracted from the fat samples were low in quality, and reduced in quantity. Thus, we hypothesized that there was an effect of DNase treatment on RNA quantity and quality from the subcutaneous adipose tissue samples. We aimed to optimize the method in RNA extraction by excluding the DNase treatment, and compare between the DNase and non-DNase treated RNA. We also determine if the non-DNase treated RNA producing a reliable outcome using the RT-qPCR, thus could be applied for our subsequent experiments.

3.5.1.2 Material and Methods

Six subcutaneous adipose tissue samples, were randomly selected and extracted for RNA irrespective of the treatment. The RNA pellets were dissolved in 40 µl of RNase-free water and the measurements of the RNA concentration were taken before commencement of the DNase treatment. The DNase treatment was carried out. The measurements of total RNA concentration were re-evaluated after the DNase treatment. The gel electrophoresis was performed, to check the quality of the RNA before and after the DNase treatment. The detail protocols for the RNA extraction, and gel electrophoresis quantification were described in Section 3.2.

3.5.1.3 Results

After DNase treatment of the RNA extraction from subcutaneous adipose tissue samples, there was a very highly significant reduction ($P<0.001$) in the RNA concentration (Figure 3.8). Before DNase treatment, the RNAs were apparently intact, with the appearance of the 18S and 28S rRNA bands (Figure 3.9 (A)). However, we observed the smearing of the RNA bands on the gel electrophoresis after the DNase treatment, suggesting the degradation of the RNA (Figure 3.9 (B)). Surprisingly, on the gel electrophoresis, there was no significant gDNA even in the non-DNase treated RNA (Figure 3.9 (A)).

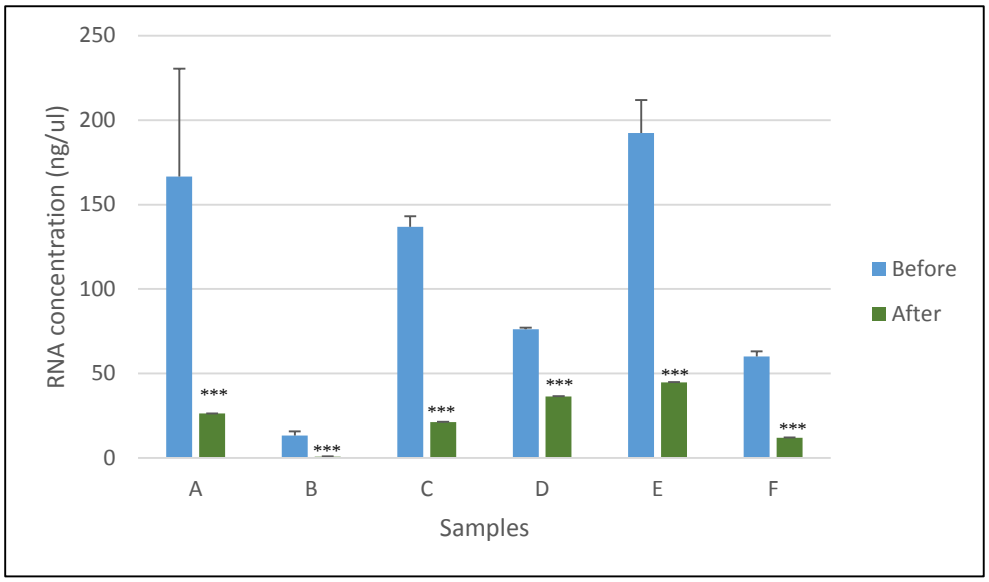


Figure 3.8: Total RNA concentration (ng/µl) before and after DNase treatment. Six individual subcutaneous adipose tissue samples (A, B, C, D, E, and F) were randomly extracted for the total RNA, and the measurement of the concentrations in triplicates were taken before and after performing the DNase treatment. Paired t-test was used to determine the effect before and after DNase treatment on total RNA concentration. The error bars are ± SEM.
*** means very highly significant different at P<0.001, as compared before and after DNase treatment, in the same sample.

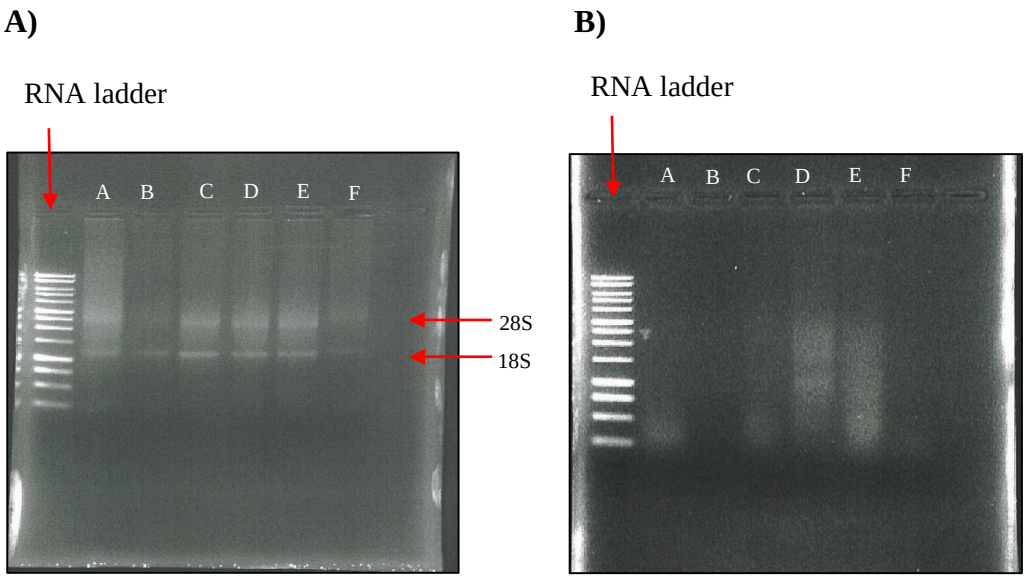


Figure 3.9: The gel electrophoresis for the quantification of RNA quality (A) before and (B) after the DNase treatment. 5 µl of RNA samples (from previous subcutaneous adipose tissue samples) (A, B, C, D, E, and F) were run through the 1% agarose gel and stained with ethidium bromide. Two bands represent rRNA bands, 28S (upper) and 18S (lower).

3.5.1.4 Discussion

The target of RNA extraction is to get a high yield of undegraded RNA. In this experiment, DNAse treatment significantly reduced RNA quantity and degraded the RNA. A review by Bustin. (2002) reported that the effect of RNases in DNase treatment, might expose the RNA towards degradation. It also has been reported that DNase treatment involving the phenol/chloroform step, would cause degradation about half of the RNA (Dotti and Bonin, 2011). The reduction of the RNA concentration after the DNase treatment also could be due to the presence of divalent ions like Mg^{2+} which is part of many DNase buffers, together with other component specially Tris, which has ability to hydrolyze diester bonds of RNA (AbouHaidar and Ivanov, 1999; Barshevskaia et al., 1987).

3.5.1.5 Conclusion

We concluded that incubation with DNase has the poor effects on the RNA quantity and quality from subcutaneous adipose tissue samples, and should be avoided during the RNA extraction. While we recommended the non-DNase treatment, the validation of the gDNA contamination should be considered, as described in the next experiment.

3.5.2 Detection of the genomic DNA (gDNA) in the non-DNase treated RNA

3.5.2.1 Introduction

As described earlier, the DNase treatment was an additional step in the RNA extraction, which selectively degraded DNA contamination, particularly gDNA that would lead to a false positive result during gene expression analysis. According to the previous pilot experiment, the DNase treatment was not included in our RNA extraction from subcutaneous adipose tissue sample, which appeared to cause degradation in our RNA samples. Thus, in this section, we hypothesized that the gDNA would present in our extracted non-DNase treated RNA samples. We have performed a test to confirm its presence, by performing RT-PCR for cDNA synthesis, with and without reverse transcriptase enzyme, and to assess the effect of the gDNA on the amplification during RT-qPCR.

3.5.2.2 Materials and Methods

Three subcutaneous adipose tissue samples were randomly selected and extracted for total RNA. The RNA pellets were dissolved in RNase free water and subjected to the gel electrophoresis for determination of RNA integrity. The cDNAs were synthesized in two separate reactions, one with RNA transcribed into cDNA with reverse transcriptase enzyme (RT) and in the other reaction the RNA was not reverse transcribed into cDNA, by excluding the reverse transcriptase enzyme (non-RT), while other reagents were included in both templates. Thereby the non-RT template would act as a control, as any amplification during qRT-PCR indicates the presence of the contaminating gDNA in the RNA. The synthesized cDNAs from both reactions were subjected to the Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) using Lightcycler 480 (Roche, UK). For each reaction, the amplification curve was examined and the crossing point (Cp) values were determined. The beta-actin gene from sheep (sheep ACTB gene) was used for the measurement. The detail protocol for the RNA extraction, gel electrophoresis, cDNA synthesis and RT-qPCR quantification were described Section 3.2.

3.5.2.3 Results

From our observations in the RNA used for this experiment, two distinct bands showing the 28S and 18S ribosomal RNA were observed and degradation of RNA was not detected after the agarose gel electrophoresis (Figure 3.10).

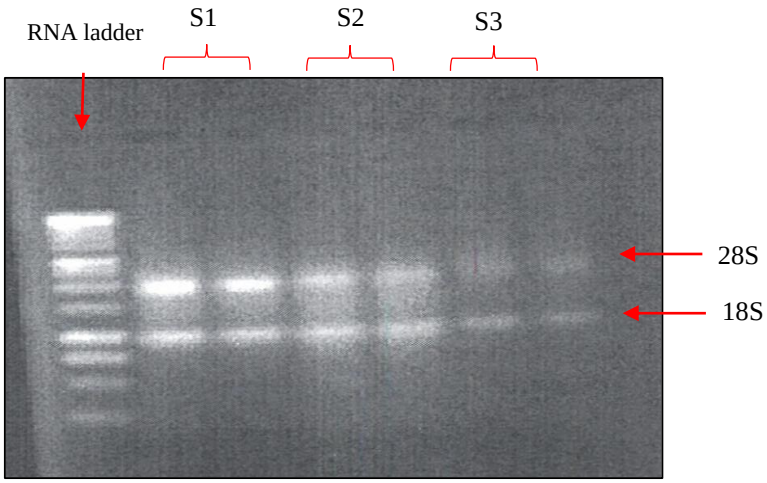


Figure 3.10: The gel electrophoresis for the determination of RNA quality. Three RNA samples from the sheep subcutaneous adipose tissue samples from the trial were run through the 1% agarose gel stained with ethidium bromide in duplicate. S1= sample 1; S2 = sample 2; S3 = sample 3. Two bands represent rRNA bands, 28S (upper) and 18S (lower).

In order to assess the amount of gDNA presence in our non-DNase treated RNA, the crossing point (Cp) values of the RNA that were reverse transcribed (RT) into cDNA were compared with non-reverse transcribed (non-RT) using qRT-PCR. For the PCR carried out on the template from the non-RT reaction, there was very late amplification of DNA (Average Cp at ~32). For the template generated from the RT reaction, Cp values were recorded at low Cp values (Average Cp at ~19). There was also a highly significant difference in the Cp value ($P<0.001$), suggesting both reactions were not overlap between each other (Figure 3.12).

A) With RT (RT) **B) Without RT (non-RT)**

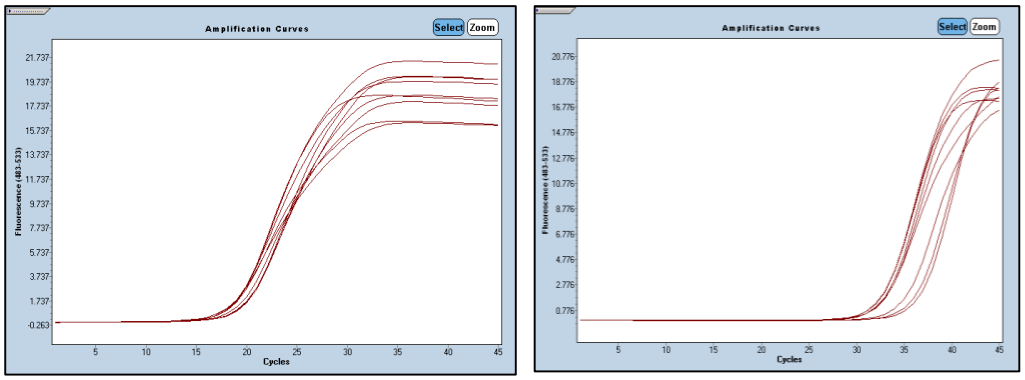


Figure 3.11: Amplification curve of the cDNA samples measured with ACTB gene as a target gene by RT-qPCR. The cDNA was synthesized from the 5 µl (the concentration of 50ng/µl) of total RNA of sheep subcutaneous fat samples after two-step RT-PCR in two reactions (A) RT and (B) non-RT, with n = 3.

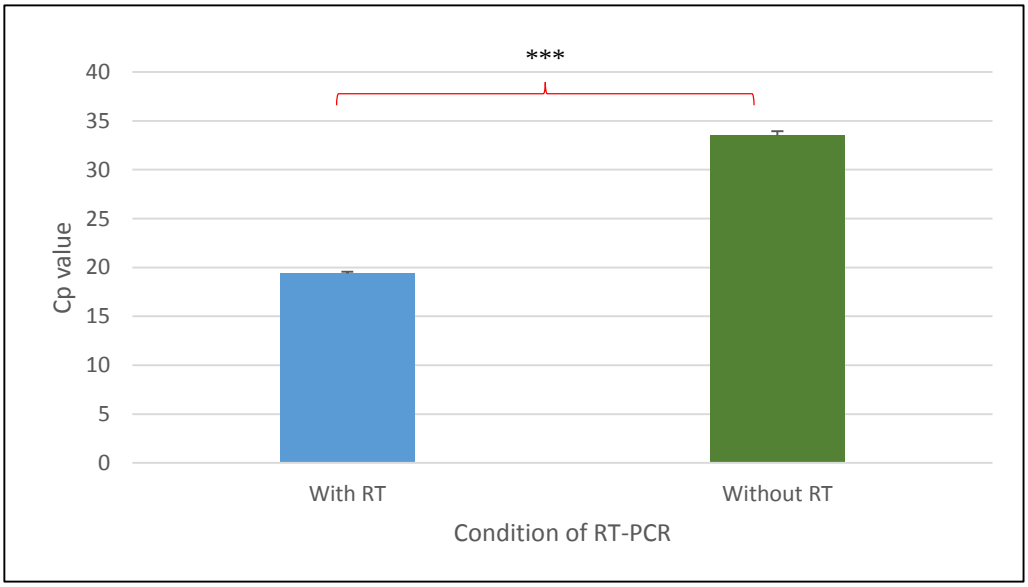


Figure 3.12: Crossing point (Cp) value of the cDNA amplification with beta actin gene (ACTB gene) as a target gene by RT-qPCR. The cDNA was synthesized by condition with or without reverse transcriptase (RT) enzyme by two-step RT-PCR, with n = 3. Paired sample student t-test was used for statistical comparison between RT and non-RT. The error bars are ± SEM.
*** means very highly significant different at P<0.001, between both conditions.

3.5.2.4 Discussion

The gel electrophoresis images showed that even when DNase treatment was not included in the purification step, there were visible intact rRNA bands (18S and 28S), and no sign of degradation, indicated the good quality of RNA. Even when

gDNA was not apparent (as similar with our previous experiment), the amplification curve generated from our non-RT samples showed late amplification, thus indicating the possible presence of the gDNA in the RNA. However, our RT cDNA showed earlier amplification, which suggesting that any gDNA concentration was low. Additionally, according to the differences in the Cp values, the non-RT samples has a Cp value ~13 cycles higher than an RT cDNA samples. This indicates the concentration of gDNA was about 2^{13}, which resulted approximately 8192-fold lower than the target sequence. So, we conclude that the gDNA presence in our RNA was approximately 0.01% (1:8192), which sufficiently negligible. This negligible amount could be reflected the undetectable gDNA on the gel electrophoresis. Laurell et al. (2012) also reported that at least five cycles (Cp value) differences between RT and non-RT reactions, which indicated approximately less than 3% (1:32) of the total amplification were produced from the gDNA, and this was generally used as limit for the gene expression quantification. If the Cp differences was less than five cycles, then the RNA should be considered to undergo DNase treatment to remove the higher contaminating gDNA (Laurell et al., 2012).

Our data showed that the quality of the RNA was sufficiently high enough for qRT-PCR even if it had been not treated with DNase. We assumed that the gDNA was presence, but negligible, and could be proceed with our subsequent experiment. Accordingly, the method (as described above) were used for the RNA extraction from the subcutaneous adipose tissue samples in the subsequent experiment.

3.5.2.5 Conclusion

From this experiment, we concluded that the lack of DNase treatment in the RNA extraction would be unlikely to lead to interference in the reaction by contaminant gDNA. However, in RNA extractions that lacked a DNase treatment step, additional test (as described above) should be performed in order to ensure the presence of gDNA would not interfere the outcome of the gene expression data.

4. EFFECTS OF METABOLIC MODIFIERS ON OVINE LIPOGENIC AND LIPOLYTIC GENE EXPRESSIONS

4.1 INTRODUCTION

The metabolic modifiers, such as beta-adrenergic agonist (BA) and growth hormone (GH) are known as repartitioning agents, as they mediate biochemical changes in nutrient use. They redirect nutrient toward lean tissue development, by targeting protein turnover in skeletal muscle as well as lipid turnover in adipose tissue but also can influence milk synthesis and secretion in mammary glands, which consequently affects the efficiency of meat and milk production (CAST, 2005). The improvement in animal body composition and growth rate by the treatment of metabolic modifiers positively affects the animal production efficiency and helps to meet the consumer's demand for lean meat due to their health concern as it potentially reduces their intake of dietary fat (Bates and Pell, 1991). The effect of BA and GH in augmenting protein synthesis and promoting myogenesis contribute to the skeletal muscle hypertrophy has been widely discussed (Lynch and Ryall, 2008; Imanaka et al., 2008; Sotiropoulos et al., 2006; Choo et al., 1992). This effect involves and stimulation of protein synthesis genes for both agents, whilst GH treatment stimulates myoblast proliferation and subsequent myofibre differentiation and BA increases skeletal muscle cross-sectional area and mass, and increases RNA concentration (Spurlock et al., 2006; Choo et al., 1992; Beermann et al., 1987).

In terms of lipid metabolism, BA and GH treatments have been previously described as inducing lipolysis and inhibit lipogenesis in intramuscular fat and adipose tissue (Mersmann, 2002; Hagström-Toft et al., 1998). However, the detailed molecular effects lipogenesis and lipolysis at a gene expression level have not been fully described, especially in ruminant species such as sheep. There were extensive studies on the indicators of lipolysis, non-esterified fatty acid (NEFA) and glycerol release upon BA and GH treatment which have been observed *in vivo* in human, rats, pigs, as well as in culture on isolated preadipocytes (Wang et al., 1999; Wabitsch et al., 1996; Peterla and Scanes,

1990; Yang and McElligott, 1989). In addition, these findings have also been supported by description of effects on specific lipogenic and lipolytic enzymes and associated factors (such as FAS, ACC, HSL, SREBP-1, and GLUT-4) (Halsey et al., 2011; Reiter et al., 2007; Langfort et al., 1999; Donkin et al., 1996; Lanna et al., 1995; Liu et al., 1994). However, little work has been carried out in sheep. In sheep, BA treatment has been shown to stimulate NEFAs and glycerol release, but there has been limited investigation of molecular mechanisms involved (Johnson et al., 2014; Mersmann, 1998). The majority of the research has focused on the effects of the metabolic modifiers on adipose tissue and the impact of these agents on reducing the deposition of fat in this tissue, thereby decreasing the fat in the animal carcass. In production animals, the skeletal muscle, adipose depots and liver are metabolically important in determining fat deposition and turnover, which then has a commercial impact on the value of the carcass. Although decreased fat deposition in the adipose depots is a commercially inefficient use of nutrients and consumers are concerned about the health implications of too much fat in their diets, a degree of fat deposition in skeletal muscle is considered desirable as it is associated with good meat quality and is known as "marbling" (Listrat et al., 2016). Therefore, although metabolic modifiers do increase the efficiency of animal production, they tend to have a negative effect on meat quality (Dunshea et al., 2016; Dunshea et al., 2005). By gaining a better understanding of the impact of metabolic modifiers on fat turnover in skeletal muscle in relationship to the other tissues that are involved in fat metabolism, it may be possible identify if the application of these agents, or other manipulations of carcass composition, can be modified to ensure there is not large detrimental effect on meat quality.

In this study, we hypothesized that the metabolic modifiers, BA and GH, exert effects toward lipid deposition mechanism in sheep, in terms of lipogenesis and lipolysis. Thus, we aimed to determine the effects of the metabolic modifier agents on the gene expression of genes associated with lipogenesis and lipolysis in sheep in the tissues associated with nutrient deposition and repartitioning, skeletal muscle, liver and adipose tissue. The effects of the metabolic modifiers BA (cimaterol) and recombinant bovine growth hormone (bGH) were examined on sheep treated at 120 days (D120) group. This age was chosen as this is the

point in sheep growth is at the maturation phase, where there is an increased deposition in body fat and a slowing of lean tissue growth (Hemmings et al., 2015). In the parts of the world where these metabolic modifiers are used commercially, they are administered for around 28 days (National Research Council, 1994). In this study, we examined the effects of these agents after administration for 6 days on sheep. The previous work in our labs had demonstrated that key metabolic changes energy metabolism enzymes were observed at this time point, which were associated with a shift to a faster fibre type and a detectable increase in muscle mass (Hemmings et al., 2015). Therefore, this study sought to determine whether the clear changes in muscle mass were also associated with altered expression of genes involved in fat metabolism, not only in muscle but also other the tissues, predominantly associated with the metabolism of this nutrient.

4.2 MATERIALS AND METHODS

The materials and methods for the BA and GH treatments on the sheep including the determination of carcass characteristics, fatty acids profile quantification, metabolomics analysis, lipogenic and lipolytic gene expressions analysis, and statistical methods were described in Section 3.2.

4.3 RESULTS

4.3.1 Effects of metabolic modifiers on sheep carcass characteristics

There was no difference of the initial body weight of the sheep. After administration of BA or GH for six days at 120 days of age, there was no significant difference in sheep body weight (Figure 4.1). This probably might be due to the relatively short treatment time of BA and GH compared to the 28-day treatment used when these agents are used commercially. However, the treatment of GH significantly increased the liver weight ($P<0.05$) (Figure 4.2), whilst there was no effect of BA or GH on the heart and kidney weight.

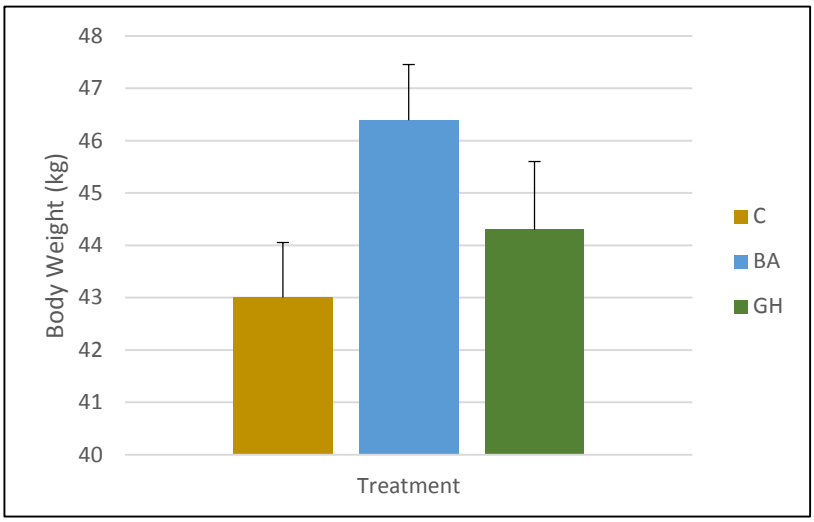


Figure 4.1: Effects of six-day treatment of BA and GH on sheep body weight. There was no significant difference between treatments, using ANOVA. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).

In muscles, BA treatment significantly increased (P<0.05) the *suprasupinatus* (SS) and *semitendinosus* (ST) weights, whilst there was a trend for an increased in *vastus lateralis* (VLAT) weight (P = 0.058). There was no effect of both treatments on *longissimus dorsi* (LD) and *vastus intermedius* (VIN) (Figure 4.3). Meanwhile, there was a very highly significant (P<0.001) reduction of LD muscle glycogen concentration by BA treatment, as relative to the control (Figure 4.4).

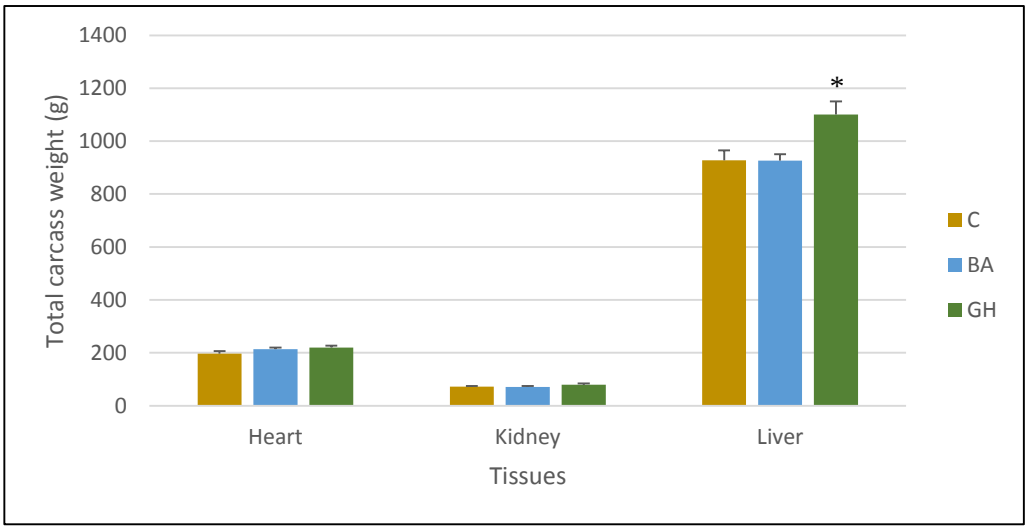


Figure 4.2: Effects of six-day treatment of BA and GH on tissue carcass weight of sheep heart, kidney, and liver. The ANOVA was used for statistical comparison in the same tissues, not between tissues. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at P<0.05, as relative to control by Dunnett's test.

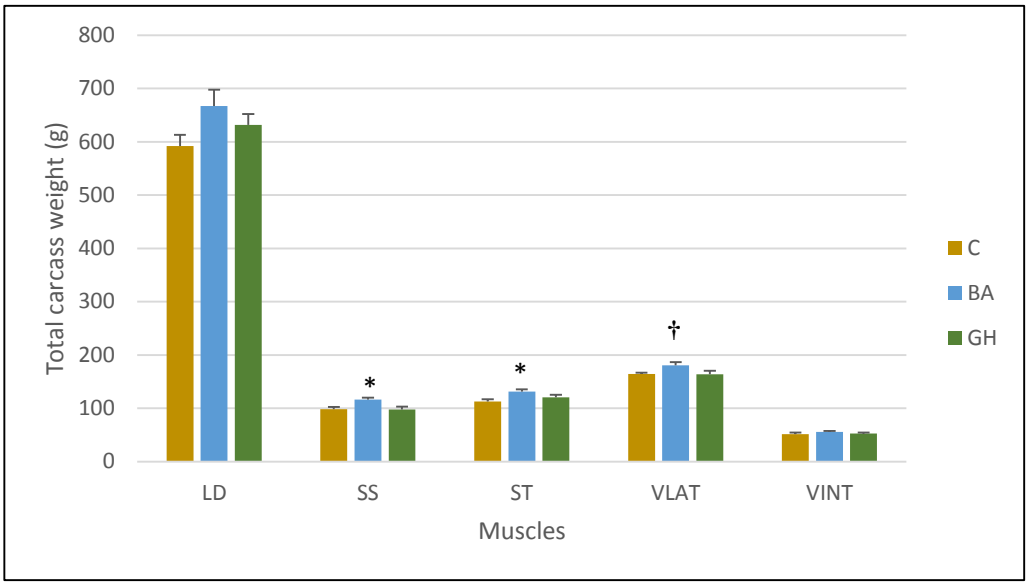


Figure 4.3: Effects of six-day treatment of BA and GH on total weight of sheep LD, SS, ST, VLAT and VIN muscles. The ANOVA was used for statistical comparison in the same muscle. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at P<0.05, as relative to control by Dunnett's test.
† means trend at P<0.1, as relative to control by Dunnett's test.

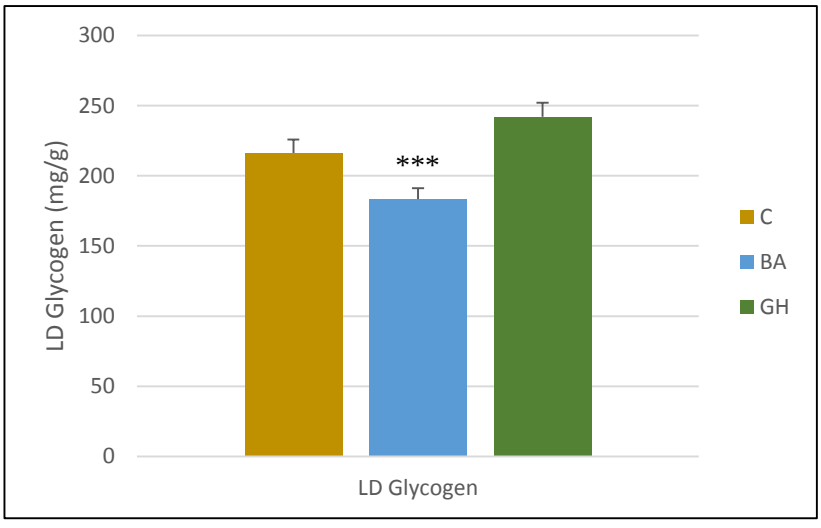


Figure 4.4: Effects of six-day treatment of BA and GH on LD muscle glycogen. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
*** means very highly significant different at P<0.001, as relative to control by Dunnett's test.

4.3.2 Effects of metabolic modifiers on sheep plasma IGF-1

As expected, the level of IGF-1 (ng/ml) was very highly significantly increased ($P<0.001$) in the plasma of the sheep treated with GH (Figure 4.5). This plasma IGF-1 level indicated the level of circulating growth factors that can act via endocrine or autocrine/paracrine pathways to mediate its effect were stimulated by the GH treatment.

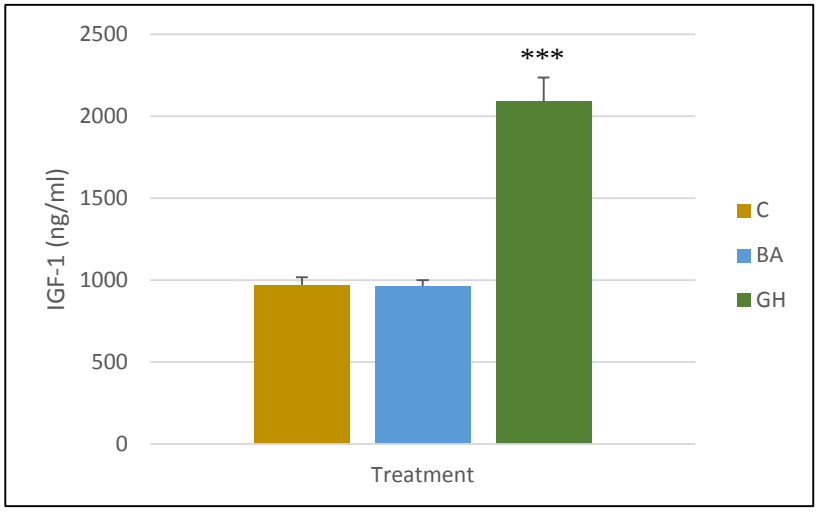


Figure 4.5: Effects of six-day treatment of BA and GH on sheep plasma IGF-1 level. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
*** means very highly significant different at $P<0.001$, as relative to control by Dunnett's test.

4.3.3 Effects of metabolic modifiers on lipid profile of sheep subcutaneous adipose and SS muscle tissues

According to previous studies, the BA and GH treatment appeared to alter lipid deposition in the tissues especially in the adipose tissue (Chaves et al., 2013; Sakharova et al., 2008; Mersmann, 2002; Yang and McElligott, 1989). However, there is no clear evidence reporting the influence of these agents on lipid composition by the use of lipid profile analysis techniques. According to the carcass characteristics, there was a significantly heavier of SS muscle from the sheep treated with BA in relative to the control, with no significant effect of GH. Thus, this sample was subjected to lipid profiling to determine whether there will be any changes in lipid composition in the muscle. Likewise, the subcutaneous

adipose tissue sample was also subjected to extraction and profiling to determine the effects of these agents on this representative fat depot.

The lipid content in the muscle and adipose tissue was demonstrated to be reduced by the treatment of metabolic modifiers (Dunshea et al., 2005). Thus, we focused on the effects of the BA and GH in altering the composition of lipid profile in the muscle and subcutaneous adipose tissue. The lipid profiling analysis indicated there was also no effect of both treatments on the fatty acid composition relative to the control in SS muscle (Table 4.1). However, in SC sample, the BA treatment significantly reduced ($P<0.05$) the palmitic acid (C16:0) composition (Table 4.2). In addition, the BA treatment also tended ($P = 0.058$) to increase the unsaturated fatty acid to saturated fatty acid (unsat/sat) ratio in the SC sample, compared with the control, while both treatments did not appear to have any effect on the unsat/sat ratio in the SS muscle (Figure 4.6).

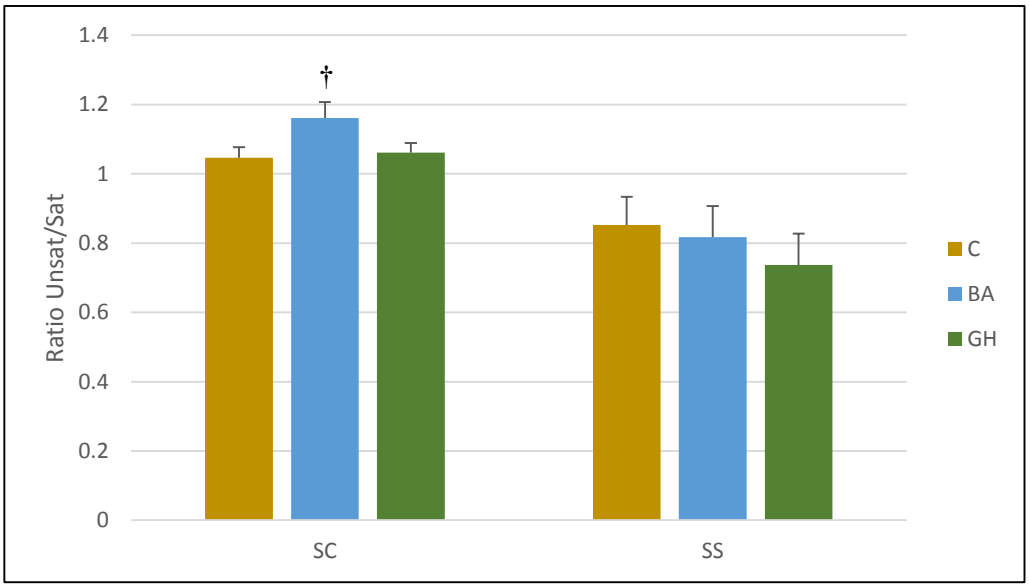


Figure 4.6: Effects of six-day treatment of BA and GH on total fat in subcutaneous adipose tissue (SC) and *suprasupinatus* muscle (SS) samples. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
† means trend at $P<0.1$, as relative to control by Dunnett's test.

Table 4.1: Fatty acid profile of the SS muscle in sheep treated with BA and GH for 6 days

Treatment	C14:0 (%)	C16:0 (%)	C16:1 (%)	C17:0 (%)	C18:0 (%)	C18:1n9t (%)	C18:1n9c (%)	C18:2n6c (%)	C18:3 (%)
C	2.85	30.03	1.85	1.28	21.14	2.03	39.81	1.00	ND
BA	2.48	31.42	1.89	1.40	20.89	2.34	38.93	0.67	ND
GH	2.61	32.46	1.52	1.26	22.77	1.82	36.78	0.79	ND
SED	0.4167	2.718	0.3996	0.2602	2.207	0.4272	3.289	0.5144	
P value	0.657	0.659	0.597	0.845	0.654	0.481	0.633	0.799	

Units = % fatty acids of total fatty acids; SED = standard error of difference of the mean, ND = not detected
The ANOVA was used for statistical comparison between the treatment, not between individual fatty acids (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone) in *suprasupinatus* muscle (SS)

Table 4.2: Fatty acid profile of the SC sample in sheep treated with BA and GH for 6 days

Treatment	C14:0 (%)	C16:0 (%)	C16:1 (%)	C17:0 (%)	C18:0 (%)	C18:1n9t (%)	C18:1n9c (%)	C18:2n6c (%)	C18:3 (%)
C	2.99	25.44	2.46	1.69	18.85	2.91	43.22	2.45	ND
BA	2.75	23.34*	2.47	1.90	18.47	3.47	44.82	2.77	ND
GH	3.09	25.22	2.36	1.79	18.49	2.95	44.06	2.04	ND
SED	0.252	0.783	0.2461	0.2624	1.2558	0.401	0.8439	0.3927	
P value	0.4	**<0.05**	0.885	0.712	0.946	0.306	0.187	0.195	

Units = % fatty acids of total fatty acids; SED = standard error of difference of the mean, ND = not detected
The ANOVA was used for statistical comparison between the treatment, not between individual fatty acids (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone) in subcutaneous adipose tissue (SC) samples.
*means significant different at P<0.05, as relative to control by Dunnett's test.

4.3.4 Metabolomics analysis of sheep plasma

In this analysis, the sheep plasma samples (all treatments), were analysed by Metabolon Inc. (USA), and based on their protocols using GC/MS and LC/MS platforms. The biochemical profiles were identified and quantified according their standard databases. The units were displayed as scaled density, in which the original area values have been re-scaled to have median equal to 1.

4.3.4.1 Effects of metabolic modifiers on plasma glycerol and monoacylglycerol concentrations

Triacylglycerol is a storage form of lipid. During lipolysis, the triacylglycerol breakdown produces glycerol and NEFAs, which are the marker for lipolysis. In both BA and GH treated lambs, the plasma glycerol level was significantly increased ($P<0.05$) (Figure 4.7). Another metabolite, 1-oleoylglycerol, the oleoyl form of monoacylglycerol which is produced by the breakdown of diacylglycerol during lipolysis, was also significantly increased ($P<0.05$) after the BA treatment (Figure 4.7). This data suggested that both treatments induced fat breakdown.

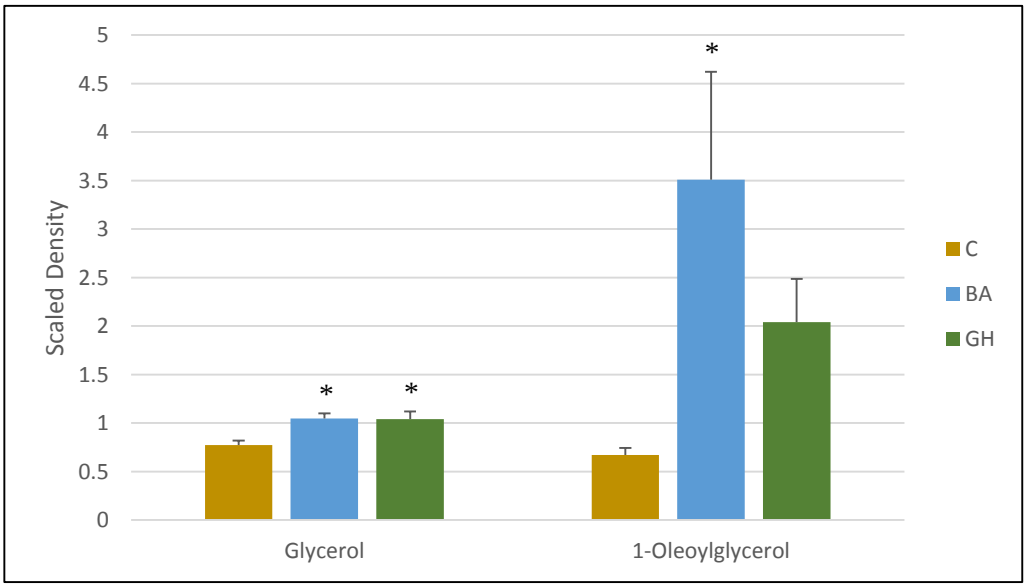


Figure 4.7: Effects of six-day treatment of BA and GH on glycerol and 1-oleoylglycerol concentrations. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at $P<0.05$, as relative to control by Dunnett's test.

4.3.4.2 Effects of metabolic modifiers on plasma non-esterified fatty acids (NEFAs) concentration

It has been reported that GH is one of the lipolytic agents, which produce a significant effect on the plasma composition, particularly on the NEFAs concentration. In terms of the essential fatty acid, as expected, the GH treatment significantly increased ($P<0.05$) the essential polyunsaturated fatty acids (PUFA), linoleate (C18:2n6) and linolenate (C18:3n3 or 3n6, as the analysis cannot distinguished between n-3 and n-6 fatty acids) (Figure 4.8) as compared with the control. However, none of the longer-chain essential PUFA, such as docosahexaenoate (C22:6n3) and dihomo-linolenate (C20:3n3 or 3n6, as the analysis cannot distinguished between n-3 and n-6 fatty acids) were affected by the treatments (Table 4.3). The exception was arachidonate (C20:4n6), which tended to increase ($P = 0.077$) with GH treatment (Figure 4.8).

The GH treatment also resulted in a significant increase in circulating saturated fatty acids (SFA) concentration. There was a significant increase ($P<0.05$) in plasma concentration of medium-chain SFA, such as caprate (C10:0) and trend ($P = 0.070$) to increase laurate (C12:0) concentration (Figure 4.9(A)). The long-chain SFA, such as myristate (C14:0), palmitate (C16:0) were also significantly increased ($P<0.05$) as well as a very highly significant increase ($P<0.001$) in plasma stearate (C18:0) concentration in GH-treated sheep compared to the control (Figure 4.9(B)).

In addition, GH treatment significantly increased the concentration of monounsaturated fatty acids (MUFA) myristoleate (C14:1n5), palmitoleate (C16:1n7), oleate (C18:1n9) ($P<0.05$), whilst there was a highly significant increase in vaccenate (C18:1n7) concentration ($P<0.01$) (Figure 4.10).

Overall, the treatment of GH, but not BA, for 6 days in lambs, stimulated an increased in circulating NEFA concentration, which are the marker for the lipolysis. Taken together with the induction of another markers, glycerol and monoacylglycerol level, both treatments induced lipolysis, but the effects were much more pronounced in GH treatment.

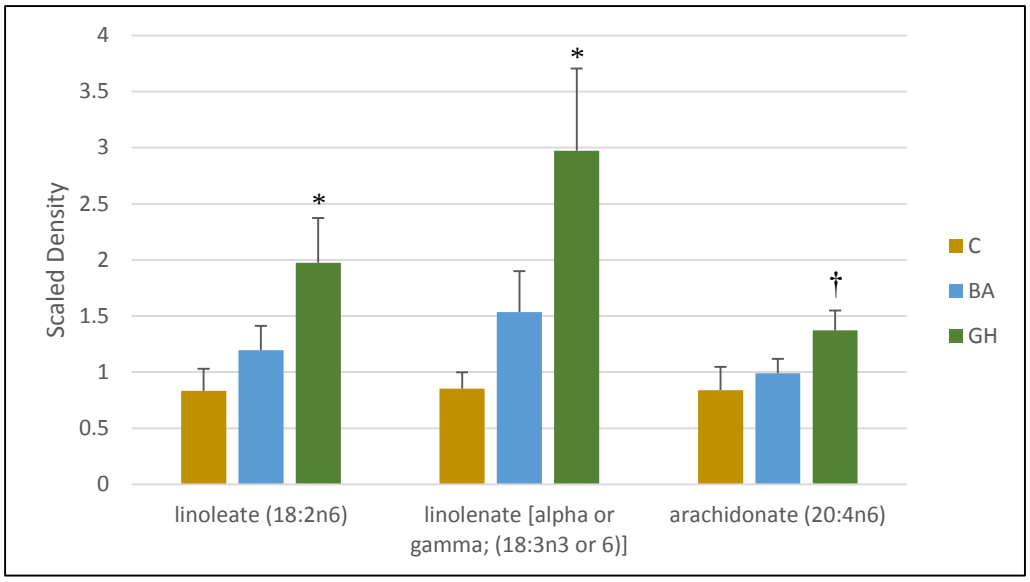


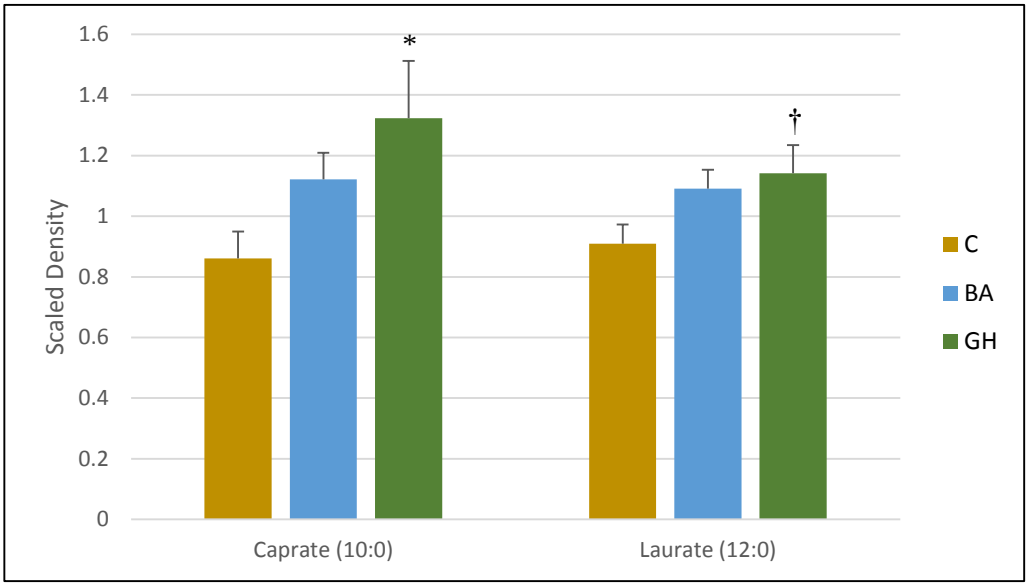
Figure 4.8: Effects of six-day treatment of BA and GH on essential polyunsaturated fatty acids; linoleate, linolenate and arachidonate concentrations. The metabolomics analysis cannot be distinguished between n3 and n6 fatty acids. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at P<0.05, as relative to control by Dunnett's test.
† means trend at P<0.1, as relative to control by Dunnett's test.

Table 4.3: Effects of six-day treatment of sheep with BA and GH on plasma dihomo-linolenate and docosahexanoate concentrations

Treatment	n	dihomo-linolenate (20:3n3 or n6)	docosahexanoate (22:6n3)
C	11	1.05 ± 0.34	2.21 ± 1.16
BA	10	1.14 ± 0.18	1.63 ± 0.53
GH	10	1.60 ± 0.29	1.96 ± 0.66
P value		0.351	0.892

Units = Scaled Density of the metabolite concentration ± SEM, The ANOVA was used for statistical comparison (C (Control), BA (beta-agonist), GH (growth hormone)).

A)



B)

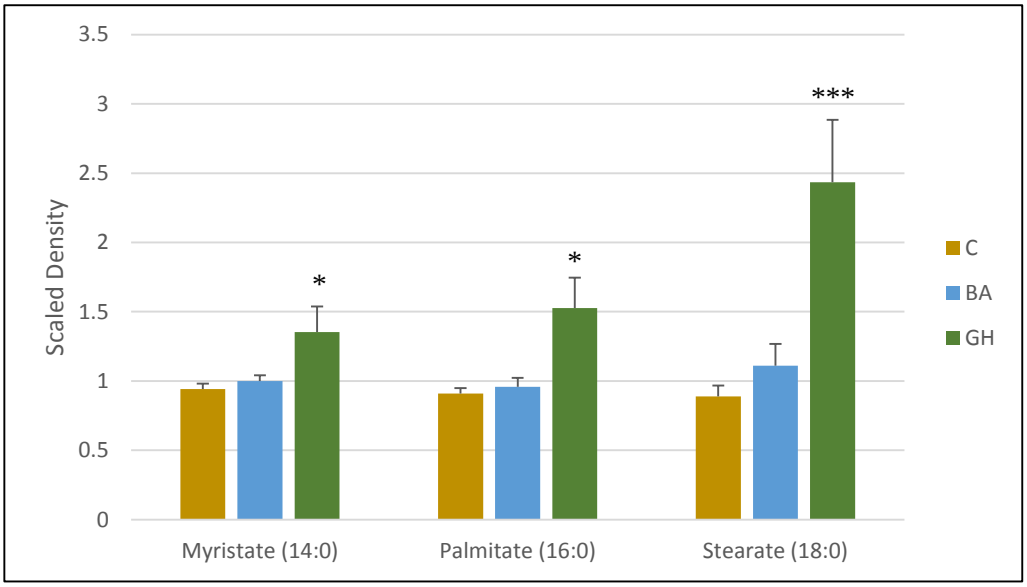


Figure 4.9: Effects of six-day treatment of BA and GH on saturated fatty acid concentrations (A) medium-chain saturated fatty acids; caprate and laurate, and (B) long-chain saturated fatty acids; myristate, palmitate and stearate. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at P<0.05, as relative to control by Dunnett's test.
*** means very highly significant different at P<0.001, as relative to control by Dunnett's test.
† means trend at P<0.1, as relative to control by Dunnett's test.

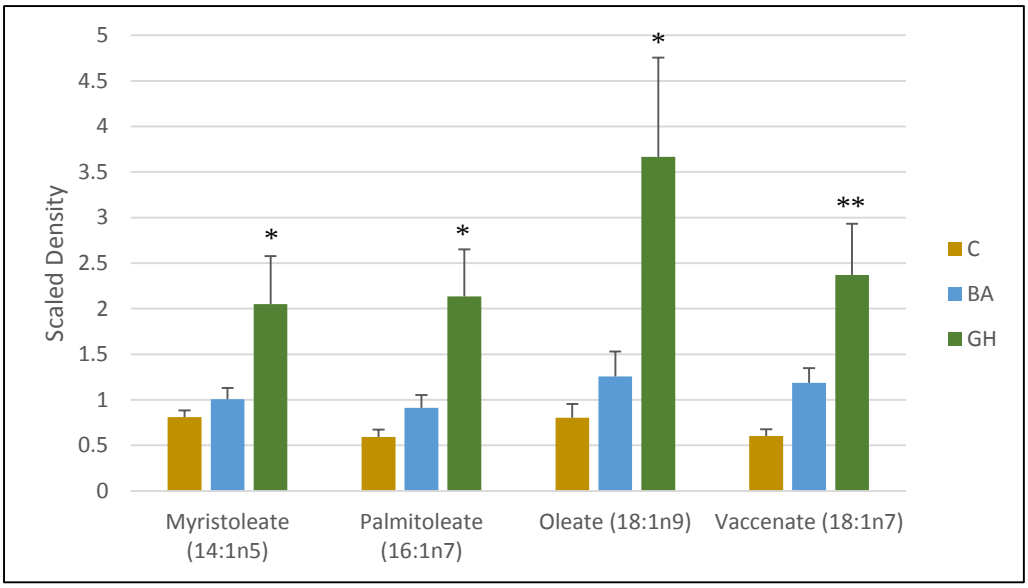


Figure 4.10: Effects of six-day treatment of BA and GH on long-chain monounsaturated fatty acid concentrations; myristoleate, palmitoleate, oleate, and vaccenate. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at P<0.05, as relative to control by Dunnett's test.
** means highly significant different at P<0.01, as relative to control by Dunnett's test.

4.3.4.3 Effects of metabolic modifiers on plasma cholesterol concentration

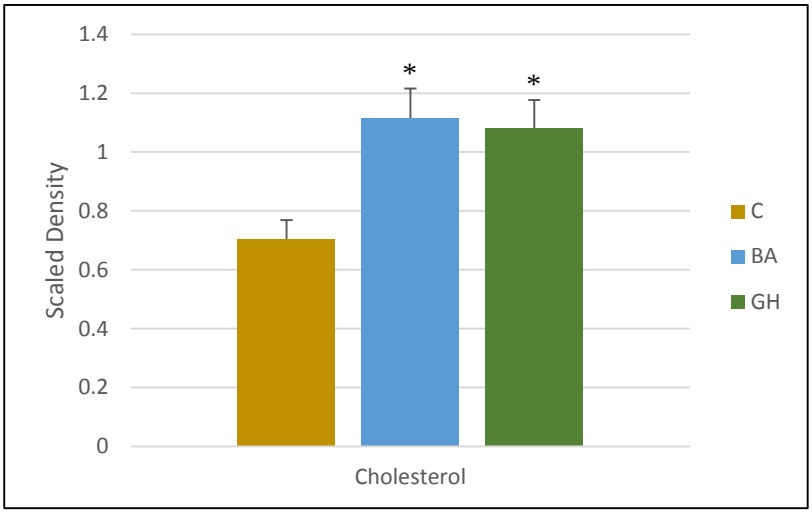
Cholesterol in the bloodstream is generated by the hydrolysis of cholesteryl esters in various tissues which is, mediated by lipolytic enzyme the hormone sensitive lipase (HSL) (Zechner et al., 2012). According to Figure 4.11(A), the plasma cholesterol in both BA and GH treated sheep were significantly higher (P<0.05), as compared with the control. This further suggest that both treatments lead to lipolysis.

4.3.4.4 Effects of metabolic modifiers on ketone bodies concentration

In the fasted state or when there is a low concentration of plasma insulin, adipose tissue mobilizes the breakdown of TAG resulting in an accumulation of plasma NEFAs. When there is an excess accumulation of NEFAs in the circulation, the liver will oxidise these to acetyl-CoA. When this metabolic intermediate accumulates due to the incapacity to oxidise it via the tricarboxylic acid (TCA) cycle, acetyl CoA is converted into ketone bodies. These have a role of acting as

an alternative source of energy in peripheral tissues and are involved in glucose sparing, particularly during fasting. The ketone bodies are categorized into acetoacetate, 3-hydroxybutyrate (or β-hydroxybutyrate) and acetone. Their appearance indicates that high levels of NEFAs are present and that lipid oxidation in the liver is elevated. The administration of GH, but not BA, significantly increased ($P<0.05$) the circulating 3-hydroxyacetate concentration, as compared with control, suggested that GH treatment stimulated the lipid oxidation (Figure 4.11(B)).

A)



B)

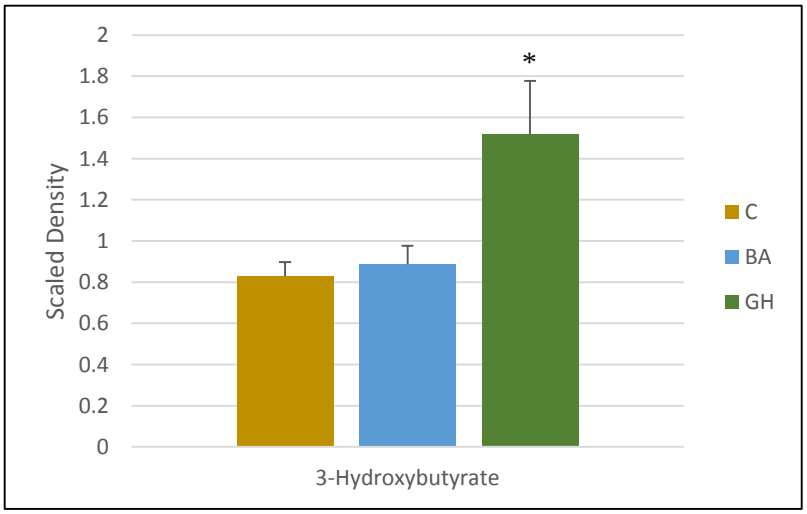


Figure 4.11: Effects of six-day treatment of BA and GH on (A) cholesterol and (B) 3-hydroxybutyrate concentrations. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at $P<0.05$, as relative to control by Dunnett's test.

4.3.4.5 Effects of metabolic modifiers on plasma glucose and non-carbohydrate carbon metabolites

The production of glucose can utilise several precursors including pyruvate, glycerol (from the lipid catabolism), α-keto acids (from the amino acids metabolism) and lactate (via Cori cycle), which will provide the carbon sources for glucose synthesis. According to the Figure 4.12, there was a very highly significant increase in plasma glucose in GH but not BA treated sheep ($P<0.001$). The GH treatment also significantly increased the plasma pyruvate concentration ($P<0.05$), while there was a trend for an increase in the plasma lactate concentration with the BA treatment ($P = 0.081$).

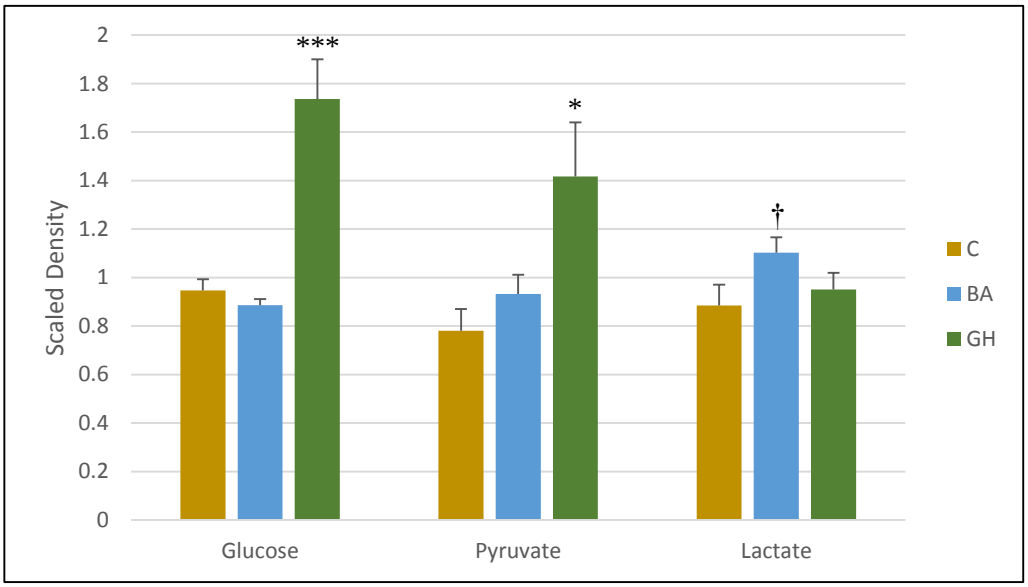


Figure 4.12: Effects of six-day treatment of BA and GH on plasma glucose, pyruvate and lactate concentrations. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at $P<0.05$, as relative to control by Dunnett's test.
*** means significant different at $P<0.001$, as relative to control by Dunnett's test.
† means trend at $P<0.1$, as relative to control by Dunnett's test.

4.3.4.6 Effects of metabolic modifiers on plasma glucogenic and ketogenic amino acid concentrations

Amino acid metabolism is one of the important aspect in the gluconeogenesis, for the formation of several intermediates. This amino acids can be categorized as glucogenic, ketogenic or both based on their intermediate products (Table 4.4). The glucogenic amino acids metabolism yields pyruvate or one of the TCA cycle intermediates, while ketogenic amino acids yields acetoacetate, which is one of the ketone bodies, and not directly involved in the formation of glucose.

Table 4.4: Classification of amino acids

Type	Non-essential	Essential
Glucogenic	Alanine	Histidine
	Arginine	Methionine
	Asparagine	Threonine
	Aspartate*	Valine
	Cystein*	
	Glutamate	
	Glutamine	
	Glycine	
	Proline	
	Serine	
Ketogenic	-	Leucine
		Lysine
Both Glucogenic and Ketogenic	Tyrosine	Isoleucine
		Phenylalanine
		Tryptophan

*not measured in this study

Following treatment with the metabolic modifiers BA and GH, it was clear that the former had an effect on increasing muscle hypertrophy whilst the latter did not. In order to increase muscle weight, it would be expected that there would be an increase in protein synthesis in muscle which would require the essential amino acids to be provided. However, treatment with GH resulted in non-

significant change in muscle weights. From the metabolomics examination, plasma GH clearly had a greater effect of increasing circulating glycerol and NEFAs than BA, and this increase in plasma NEFAs was associated with an increased production of ketone bodies presumably from the liver. In addition, there was an increase in plasma glucose concentrations in the GH treated sheep suggesting either an increased insulin resistance in the peripheral tissues resulting in a reduced plasma glucose clearance or an increased glucose synthesis by gluconeogenesis or a combination of both of these effects. Therefore, the consideration of the metabolomics analysis of plasma amino acids was done based on classifying the amino acids into groups which were aligned with their glucogenic potential.

4.3.4.6.1 Effects of metabolic modifiers on plasma non-essential and essential glucogenic amino acid concentrations

The plasma concentration of non-essential glucogenic amino acids such as alanine, arginine, asparagine and glutamate, were not affected by either treatment (Table 4.5). However, there was a significant reduction ($P<0.05$) in glutamine and glycine concentrations in the sheep plasma treated with GH (Figure 4.13). In addition, the GH treatment also tended to reduce plasma serine and proline concentrations ($P = 0.065$ and $P = 0.078$, respectively) (Figure 4.13). The BA treatment had no effect on any plasma non-essential glucogenic amino acid concentrations.

Meanwhile, for the essential glucogenic amino acids, the histidine concentration was significantly ($P<0.05$) reduced by the effect of BA treatment, as compared with the control (Figure 4.13). Both BA and GH treatments significantly reduced ($P<0.001$ and $P<0.05$, respectively) the concentration of methionine (Figure 4.13). Thus, this appeared that the BA treatment had the strong effects on essential glucogenic amino acid concentrations. However, both treatments had no effect on plasma threonine and valine concentrations (Table 4.5).

Table 4.5: Effects of six-day treatment of sheep with BA and GH on plasma alanine, arginine, asparagine, glutamate, threonine and valine concentrations

Treatment	n	Alanine	Arginine	Asparagine	Glutamate	Threonine	Valine
C	11	1.07 ± 0.07	1.04 ± 0.06	1.18 ± 0.09	0.91 ± 0.05	1.04 ± 0.06	1.15 ± 0.05
BA	10	0.98 ± 0.07	0.97 ± 0.04	1.03 ± 0.11	1.07 ± 0.08	0.90 ± 0.07	0.95 ± 0.05
GH	10	1.12 ± 0.10	1.16 ± 0.10	0.92 ± 0.08	1.05 ± 0.07	1.10 ± 0.15	1.10 ± 0.14
P value		0.502	0.169	0.190	0.179	0.386	0.266

The data is presented as Scaled Density of the amino acid concentrations ± SEM, The ANOVA was used for statistical comparison (C (Control), BA (beta-agonist), GH (growth hormone)).

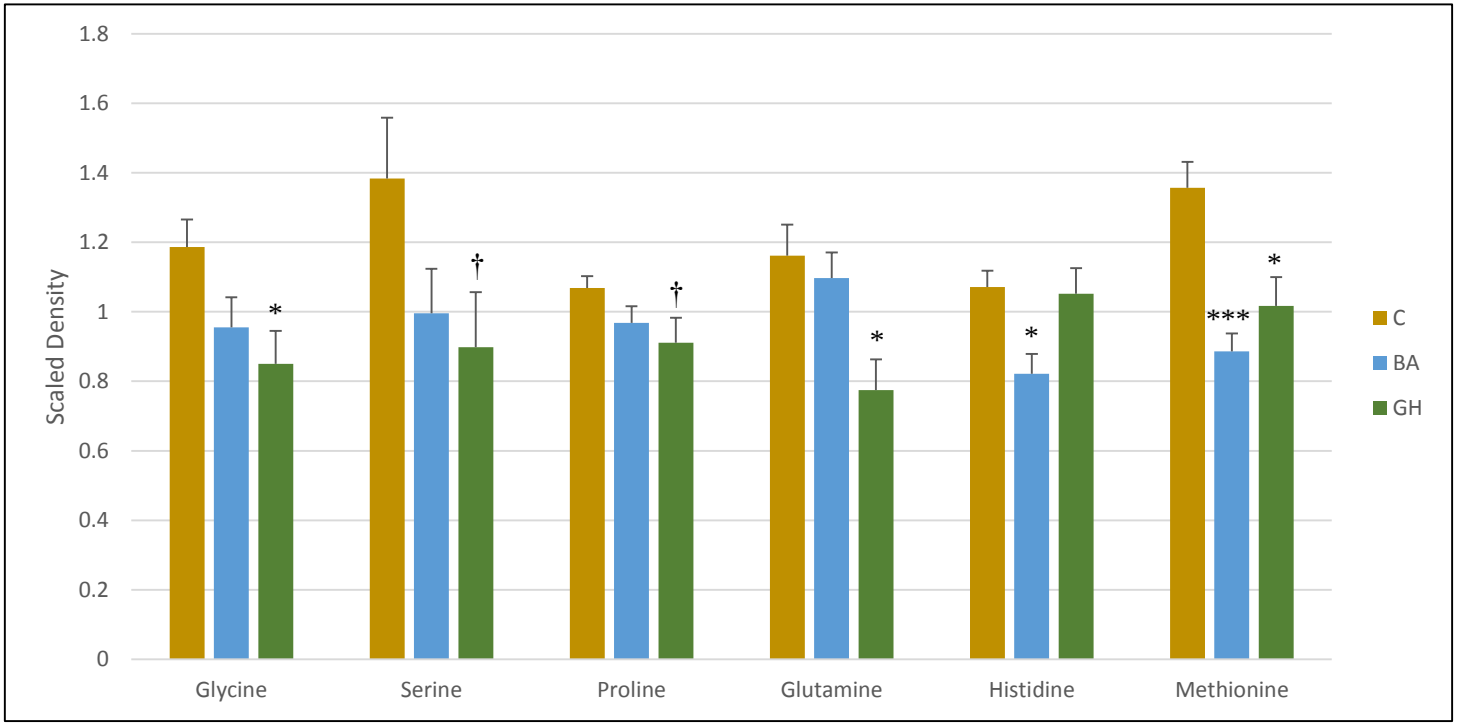


Figure 4.13: Effects of six-day treatment of BA and GH on plasma glycine, serine, proline, glutamine, histidine and methionine concentrations. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at P<0.05, as relative to control by Dunnett's test.
*** means very highly significantly different at P<0.001, as relative to control by Dunnett's test.
† means trend at P<0.1, as relative to control by Dunnett's test.

4.3.4.6.2 Effects of metabolic modifiers on plasma essential ketogenic amino acid concentrations

The plasma concentration of essential amino acids which are exclusively ketogenic, lysine and leucine were not affected by the treatment (Table 4.6).

Table 4.6: Effects of six-day treatment of sheep with BA and GH on plasma lysine and leucine concentrations

Treatment	n	Lysine	Leucine
C	11	1.09 ± 0.06	1.16 ± 0.06
BA	10	0.91 ± 0.08	0.92 ± 0.04
GH	10	1.00 ± 0.09	1.09 ± 0.15
P value		0.271	0.187

The data is presented as Scaled Density of the amino acid concentrations ± SEM, The ANOVA was used for statistical comparison (C (Control), BA (beta-agonist), GH (growth hormone)).

4.3.4.6.3 Effects of metabolic modifiers on plasma non-essential and essential glucogenic and ketogenic amino acid concentrations

For the non-essential amino acids, only tyrosine is the amino acid which is both glucogenic and ketogenic. According to Table 4.7, both treatments had no effect on plasma tyrosine concentration.

Meanwhile, for the essential amino acids, there were no significant effects of BA and GH on plasma isoleucine and phenylalanine concentrations (Table 4.7). However, there was a trend that GH treatment tend to reduce (P = 0.065) tryptophan plasma concentration, as compared with the control (Figure 4.14).

Table 4.7: Effects of six-day treatment of sheep with BA and GH on plasma tyrosine, isoleucine and phenylalanine concentrations

Treatment	n	Tyrosine	Isoleucine	Phenylalanine
C	11	1.07 ± 0.08	1.10 ± 0.05	1.14 ± 0.08
BA	10	1.07 ± 0.06	0.94 ± 0.04	1.02 ± 0.04
GH	10	1.00 ± 0.09	1.19 ± 0.16	0.98 ± 0.07
P value		0.770	0.226	0.184

The data is presented as Scaled Density of the amino acid concentrations ± SEM, The ANOVA was used for statistical comparison (C (Control), BA (beta-agonist), GH (growth hormone)).

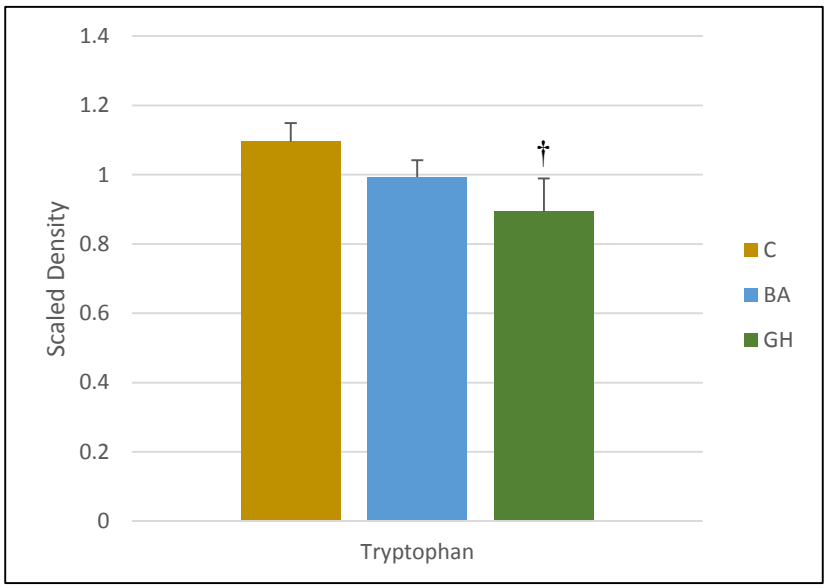


Figure 4.14: Effects of six-day treatment of BA and GH on plasma tryptophan concentration. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone). † means trend at P<0.1, as relative to control by Dunnett's test.

4.3.4.7 The effects of metabolic modifiers on the plasma amino acids metabolic product concentrations

Another indicator of the amino acid metabolism was their end products or metabolites. According Figure 4.15, GH treatment significantly ($P<0.05$) increased plasma alpha-hydroxyisocaproate, which is the end product of leucine metabolism as well as the 2-hydroxyisobutyrate concentration, which is the product from threonine and methionine catabolism. In addition, the plasma concentration of glycine metabolism, serine and threonine intermediate, beta-hydroxypyruvate, was very highly significant ($P<0.001$) increased by GH treatment. However, the plasma uroconate concentration, the metabolite of histidine metabolism tended to be reduced ($P = 0.094$) by BA treatment, but was significantly reduced by GH treatment ($P<0.05$). The plasma indoleacetate concentration, which is the product of tryptophan catabolism was also reduced by GH treatment ($P<0.05$).

Meanwhile, the alpha-hydroxyisovalerate, which is the product from leucine, isoleucine and valine metabolism, pipecolate, which is the metabolite from lysine metabolism, and 4-hydroxylphenylacetate, which is the metabolite from tyrosine metabolism, were all not affected by both treatments (Table 4.8).

Table 4.8: Effects of six-day treatment of BA and GH on plasma alpha-hydroxyisovalerate, pipecolate and 4-hydroxylphenylacetate concentrations

Treatment	n	alpha-hydroxyisovalerate	pipecolate	4-hydroxylphenylacetate
C	11	0.99 ± 0.12	2.17 ± 0.62	1.00 ± 0.10
BA	10	1.93 ± 1.05	4.38 ± 1.83	1.35 ± 0.27
GH	10	2.57 ± 0.73	1.75 ± 0.49	0.82 ± 0.11
P value		0.299	0.234	0.107

The data is presented as Scaled Density of the amino acid concentrations ± SEM, The ANOVA was used for statistical comparison (C (Control), BA (beta-agonist), GH (growth hormone)).

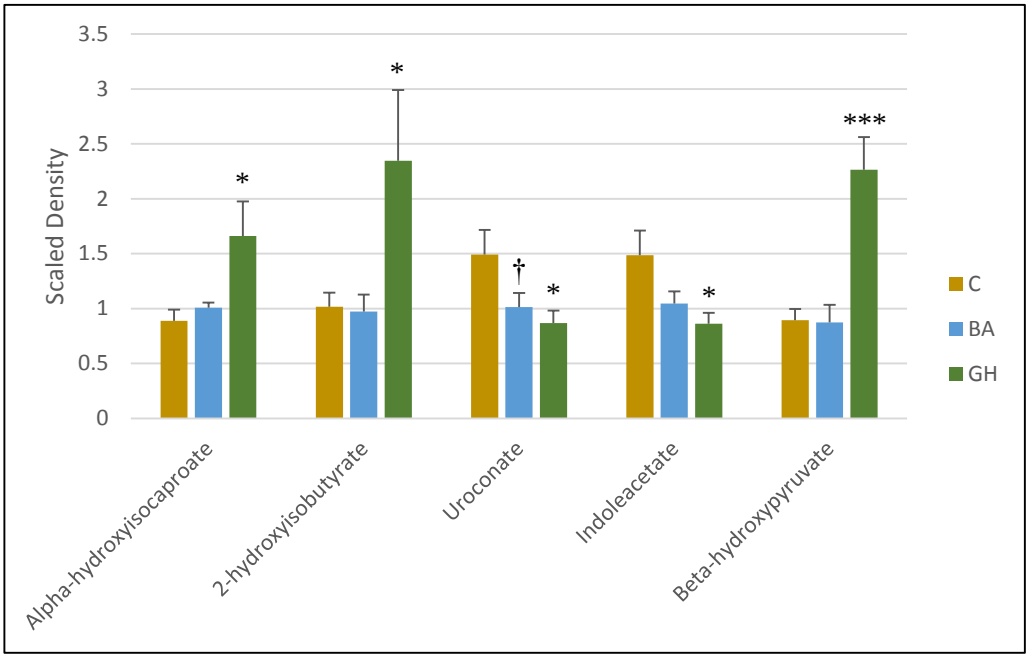


Figure 4.15: Effects of six-day treatment of BA and GH on plasma alpha-hydroxyisocaproate, 2-hydroxyisobutyrate, uroconate, indoleacetate, and beta-hydroxypyruvate concentrations. The ANOVA was used for statistical comparison. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at P<0.05, as relative to control by Dunnett's test.
*** means very highly significant different at P<0.001, as relative to control by Dunnett's test.
† means trend at P<0.1, as relative to control by Dunnett's test.

4.3.5 Effects of metabolic modifiers on lipogenic and lipolytic gene expressions

Lipogenesis (lipid synthesis) and lipolysis (lipid degradation) are regulated to maintain the homeostasis of lipid deposition. According to the above observations, the GH treatments increase the plasma NEFAs concentration. As NEFAs indicated the effects of lipolysis by GH treatment, and NEFAs could contribute to the *de novo* lipogenesis (DNL) (Saporano et al., 2015), the muscle tissues, liver and subcutaneous adipose tissue were analysed to determine the effect of both BA and GH treatments on their lipolytic as well as lipogenic activity, based to their mRNA expression level.

4.3.5.1 Determination of normalisation factors

In the gene expression analysis, the data normalisation factors are important to improve reliability by correcting errors and variability in the samples. In RT-qPCR technique, the gene expressions were normalised to the expression of the reference gene to give the corrected value. This corrected value was then analysed to summarize the mean of the individual gene expression in the samples. The important criteria is the expression of the reference gene should not be affected by the treatment. Another alternative is using oligreen. This assesses the total quantity of cDNA generated by reverse transcriptase thereby directly assessing the efficiency of the critical step in quantitative RT-PCR which dictates the quantity of cDNA template present. The value obtained for the oligreen assessment can be used to normalise the values obtained for the gene expression of a target gene, as described in Section 3.2. The synthesized cDNAs from the extracted RNAs in the RT-PCR were subjected for the determination of the normalization factor using RT-qPCR. The commonly used reference gene, beta actin (ACTB) and the oligreen, were tested and the expression for both factors in subcutaneous adipose tissue (SC), *longissimus dorsi* (LD) muscle, *suprasupinatus* (SS) muscle and liver (LI) were summarized in Table 4.9.

The ANOVA analysis of the oligreen concentration showed no significant different among the treatment across all tissues (Table 4.9 (B)), however there was a trend ($P = 0.051$) of differences in the ACTB mRNA expression in the LD muscle, due to the significant effect between BA and GH treatment (Table 4.9 (A)). This observation might interfere with the subsequent data normalisation.

Thus, the expression of all genes in this study were normalized with oligreen concentration. Previous study by Al-Doski. (2015) also showed high variability of reference gene expressions, such as ACTB, ribosomal protein large P0 (RPLP0), and cyclophilin, compared with oligreen concentration. Moreover, the choice of the oligreen as the normalisation factor in this study would also ensure the method consistency with the previous study in the gene expression study in sheep treated with BA and GH (Al-Doski, 2015).

Table 4.9: Effects of six-day treatment of sheep with BA and GH on (A) ACTB mRNA expression and (B) oligreen concentration (cDNA quantity) in subcutaneous adipose tissue (SC), *longissimus dorsi* (LD) muscle, *suprasupinatus* (SS) muscle and liver (LI)

A)

Treatment	n	SC	SS	LD	LI
C	11	0.11 ± 0.04	0.09 ± 0.01	0.14 ± 0.03	0.04 ± 0.01
BA	10	0.10 ± 0.04	0.11 ± 0.01	0.18 ± 0.02	0.04 ± 0.01
GH	10	0.07 ± 0.02	0.09 ± 0.02	0.11 ± 0.01	0.04 ± 0.01
P value		0.740	0.575	0.051	0.266

B)

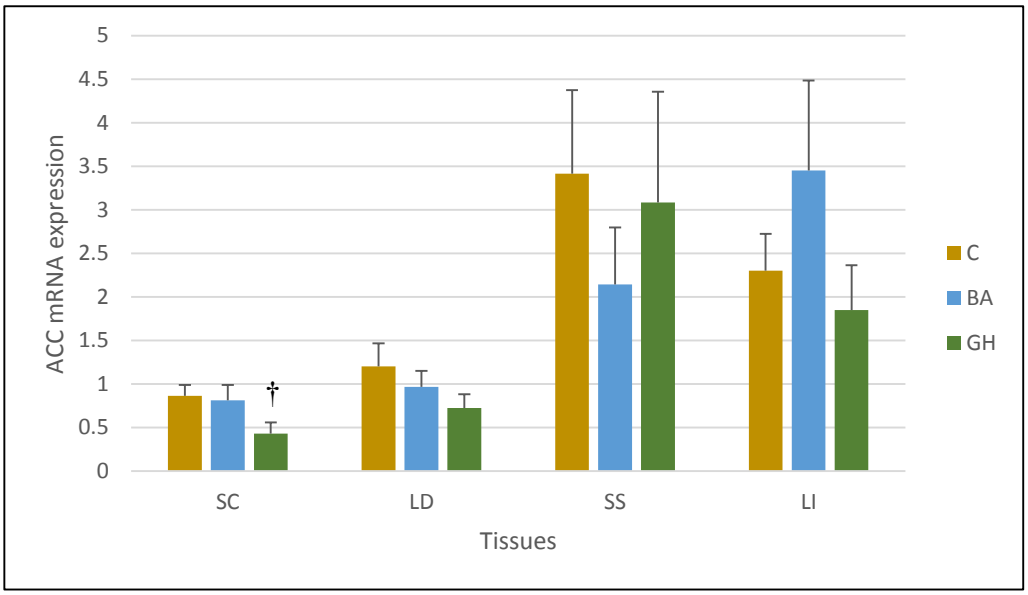
Treatment	n	SC	SS	LD	LI
C	11	0.11 ± 0.01	0.05 ± 0.00	0.14 ± 0.01	0.05 ± 0.01
BA	10	0.15 ± 0.02	0.05 ± 0.00	0.14 ± 0.01	0.05 ± 0.00
GH	10	0.12 ± 0.01	0.06 ± 0.01	0.13 ± 0.01	0.04 ± 0.01
P value		0.126	0.624	0.516	0.730

The data is presented as (A) ACTB mRNA mean concentration of the expression ± SEM and (B) oligreen concentration ± SEM, The ANOVA was used for statistical comparison (C (Control), BA (beta-agonist), GH (growth hormone)).

4.3.5.2 Effects of metabolic modifiers on the expression of genes related to lipogenesis

To determine the effect of both treatments on lipogenesis, we measured the expression level of FAS and ACC. As described earlier, both are key lipogenic genes, which involved in the biosynthesis of fatty acids. ACC catalysed the carboxylation of acetyl-CoA for the production of malonyl-CoA, while FAS catalysed the production of palmitic acid by the addition of acetyl group from acetyl-CoA. According to Figure 4.16(A), the ACC mRNA expression levels were not significantly different among the BA and GH treatments, in all tissues. However, it appeared that GH treatment showed a trend ($P = 0.084$) to reduce the ACC mRNA expression in the SC. This might suggest that the GH tended to downregulate lipogenic activity in the subcutaneous adipose tissue. Meanwhile according to Figure 4.16(B), FAS mRNA expression level were significantly downregulated ($P<0.05$) in LD muscle as well as in SS muscle ($P<0.01$) and SC ($P<0.001$) after GH treatment. The effect of BA treatment was observed to strongly downregulate FAS mRNA expression only in SS muscle ($P<0.001$). Both mRNA expressions were unaffected in liver, either treated with BA or GH. These observations suggest reduced lipogenic activity in the tissues except liver.

A)



B)

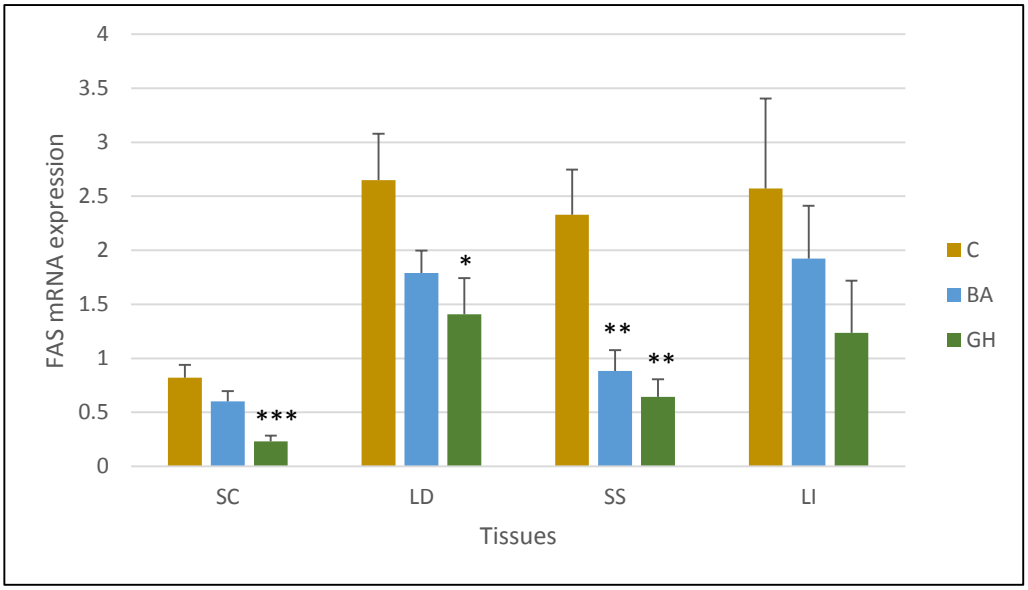


Figure 4.16: Effects of six-day treatment of BA and GH on (A) ACC mRNA expression and (B) FAS mRNA expression in SC, LD, SS and LI. The ANOVA was used for statistical comparison in the same tissues, not between tissues. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at P<0.05, as relative to control by Dunnett's test.
** means highly significant different at P<0.01, as relative to control by Dunnett's test.
*** means very highly significant different at P<0.001, as relative to control by Dunnett's test
† means trend at P<0.1, as relative to control by Dunnett's test.

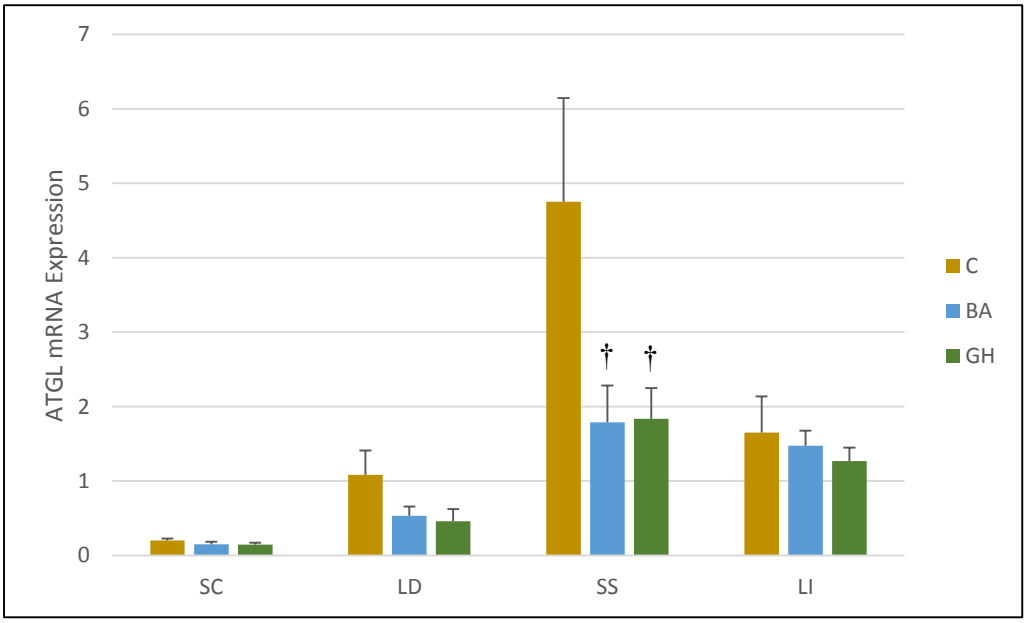
4.3.5.3 Effects of metabolic modifiers on gene expressions related to lipolysis

To determine the effect of both treatments on lipolysis, we measured the expression level of ATGL and HSL. ATGL mediates the breakdown of triglyceride into diglyceride, while HSL involved in diglyceride breakdown. According to Figure 4.17(A), the treatment with BA ($P = 0.058$) and GH ($P = 0.062$) were shown a trend to reduce ATGL mRNA expression in SS muscle, while in Figure 4.17(B), the BA treatment was tended to reduce the HSL mRNA expression level, in SS muscle as well ($P = 0.093$). This data suggested that both treatments might tend to affect the lipid turnover in the SS muscle, while the treatments in other tissues did not show any effect of lipolysis.

4.3.5.4 Effects of metabolic modifiers on adiponectin gene expression

In this study, the adiponectin was measured because of its importance in the regulation of uptake of glucose and lipid oxidation. There was a significant downregulation ($P<0.05$) of adiponectin mRNA expression level, only in SC, relative to control after GH treatment (Figure 4.18). There was no significant effect of either BA or GH treatment in adiponectin mRNA expression in LD, SS and LI.

A)



B)

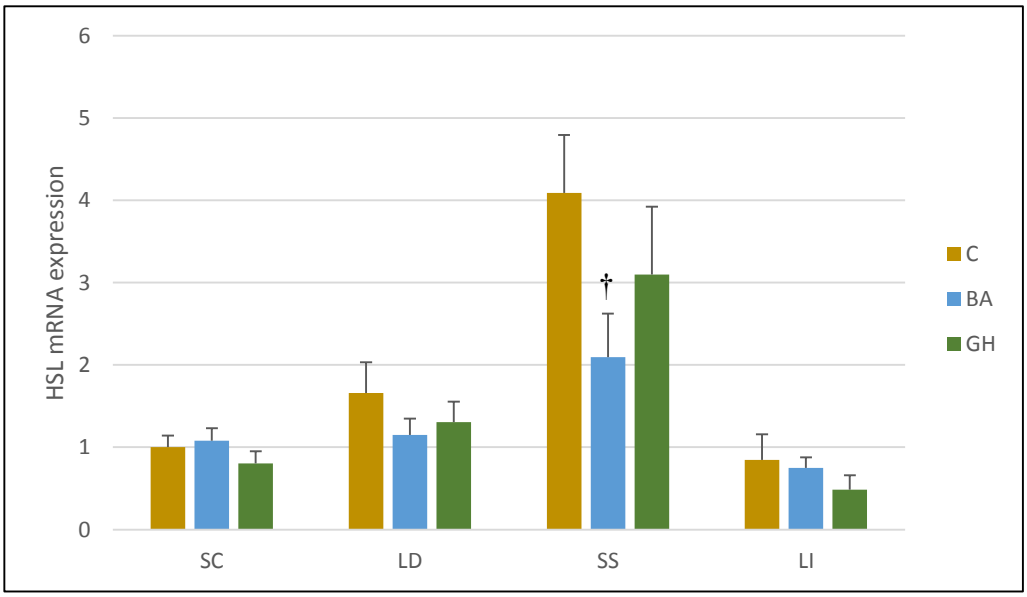


Figure 4.17: Effects of six-day treatment of BA and GH on (A) ATGL mRNA expression and (B) HSL mRNA expression in SC, LD, SS and LI. The ANOVA was used for statistical comparison in the same tissues, not between tissues. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
† means trend at P<0.1, as relative to the control by Dunnett's test.

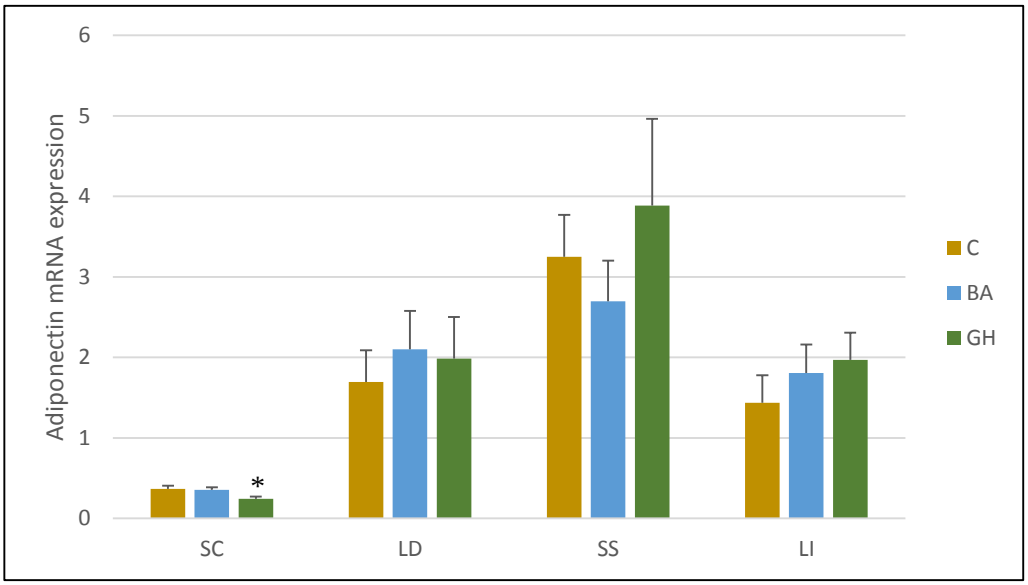


Figure 4.18: Effects of six-day treatment of BA and GH on adiponectin mRNA expression in SC, LD, SS and LI. The ANOVA was used for statistical comparison in the same tissues, not between tissues. The error bars are ± SEM. (n=11, C (Control); n=10, BA (beta-agonist), n=10, GH (growth hormone).
* means significant different at P<0.05, as relative to control by Dunnett's test.

4.4 DISCUSSION

4.4.1 Effects of metabolic modifiers on carcass characteristics in sheep

In this study, the exposure to the metabolic modifiers, BA and GH were examined to determine the effects of a relatively short period of exposure (6 days) on metabolic changes in sheep, with a focus on lipid metabolism. We found that the effects of BA and GH on the changes in carcass characteristic particularly in the muscles and liver, might be associated with the changes in lipogenesis and lipid turnover.

Commercial administration of both BA and GH is for periods of around 30 days, which is associated with significant increases in body weight (Strydom et al., 2009) and 49 days (Warriss et al., 1989). In this study, in terms of body weight, there was no significant effects of BA and GH, which might be expected, due to the short time of administration the effect of short time of treatment. However, the SS and ST muscle weights of the BA-treated lambs, were significantly increased, and VLAT muscle weight tended to be higher, compared to the control. This was in agreement with the reports, stated that the BA treatment have been positively correlated with the increase in muscle hypertrophy, which is associated with the stimulation in muscle protein synthesis, reduction in protein degradation, or a combination of both (Mersmann, 1998; Kim and Sainz, 1992). In addition, our findings regarding the BA on the muscle hypertrophic effect was remarkable, as it appeared even after short time treatment (6 days). The BA stimulated hypertrophic effect also was thought to be associated with a response to the treatment of the fast muscle fibres (Type II) rather than slow muscle fibres (Type I) (Kim and Sainz, 1992; Yang and McElligott, 1989). In this study, both ‘slow’ type muscle, SS and ‘fast’ type muscle, ST responded, which might have suggested that the effect was varied between muscles and fibre type of the muscle is not a major factor for determining muscle hypertrophy response towards the treatment (Hemmings et al., 2015). A review by Lynch and Ryall. (2008) reported that BA hypertrophic effect on muscle could be greater in fast, slow, or similar in both fibre types, while the effect being dependent on species, age of the animal, types of BA, along with the effect of mode, dosage

and frequency of treatment. They also suggested that tissue responsiveness towards BA could vary between different tissues within the same species, which might be due to different densities of receptor subtypes on each tissue (Lynch and Ryall, 2008). However other research has described the action of BA on the slow-to-fast muscle fibre transitions, which might have explained the slow fibre transition in SS to become 'fast-like' (Hemmings et al., 2015; Lynch and Ryall, 2008). The effect of BA on muscle hypertrophy also associated with the increase in cross-sectional area of the muscle, and not increase in carcass weight, which suggested the repartitioning effect of the treatment (Johnson et al., 2014; Beerman et al., 1986). The effect of BA on energy repartitioning might also contribute to increase blood flow and enhance the muscle hypertrophy process, as it may take part in nutrient and substrate availability to the muscle for protein metabolism (Al-Doski, 2015; de Almeida et al., 2012; Mersmann, 1998).

Slow-to-fast effect of BA in the muscles might also influence the increase the fast fibre proportion, resulted in the reduced muscle glycogen content. This has been reported previously by Warriss et al. (1989). This effect on glycogen might be associated with the fibre transition in the LD muscle toward a more 'fast-like' and more glycolytic. This transition would contribute to the increased sensitivity in glycogen breakdown and mobilization (Warriss, 1990; Maltin et al., 1989), reflecting capacity in utilizing glucose and lactate production (Hamby et al., 1986). In earlier studies the BA have been shown to stimulate glycogenolytic effects (breakdown of glycogen into glucose) by the conversion of glycogen phosphorylase kinase *b* (non-activated kinase) to *a* (activated kinase), while reduced effect of glycogen synthase, and increased lactate production via cAMP-mediated pathway (Levy et al., 2008; Warriss, 1990; Fiems, 1987; Richter et al., 1982) which results in glycogen mobilisation.

As describe in the literature, the role of GH is not just promoting growth, but also crucial in maintaining body composition and lipid metabolism including lipogenic and lipolytic capacity (Kawai et al., 2007). In this study, GH treatment significantly increased the liver weight, a similar effect as reported by Brameld et al. (1996). This could be due to the liver being more responsive to the GH in a short treatment period (6 days). This might due to the higher concentration of

GHR on the liver (Bates and Pell, 1991). In addition, liver is considered as a major target for GH-binding. Liver is the network centre for the transported nutrients therefore the effect of GH appears to be an effect on nutrient absorption efficiency in the liver, leading to increase in mass (Pell et al., 1990). The study on liver GHR-knockout in mice also leads to the decrease liver weight (normalization with body weight), suggesting that the liver size could be affected by the direct stimulation of GH (Sjögren et al., 2000). GH also stimulates the protein synthetic rates, as it demonstrated high liver DNA content after GH treatment (Al-Doski, 2015; Bates and Pell, 1991), which could contribute to increase liver mass.

As expected, the plasma IGF-1 concentration was also elevated after GH treatment, in line with previous studies (Govoni et al., 2004; Vestergaard et al., 2003). GH induces the secretion of IGF-1 in the liver, which is then transported via circulation and effects target tissues, or mediates the production of IGF-1 via paracrine action in other tissues (Laron, 2000).

In this study, GH treatment appeared to not affect muscle hypertrophy *in vivo*. As observed in this study and others, GH effects are thought to affect whole metabolism (such as increase liver weight, increase circulating IGF-1, increase circulating NEFAs), unlike BA which specifically affects muscle hypertrophy, in short term period (Al-Doski, 2015). In *in vitro* studies, it has been demonstrated that GH is involved in the satellite cell proliferation via activation of mechano-growth factors (MGFs) and IGF-IEa (splice IGF-1 cloned) (Imanaka et al., 2008). In GHR-knockout mice there is reduction of the cross-sectional area of the myofibre in primary cultures (Sotiropoulos et al., 2006), which suggests that GH influenced muscle growth (Bates and Pell, 1991). The increase in circulation IGF-1 by GH treatment did not mediate muscle hypertrophy effect, however it was demonstrated to be affected by the local IGF-1 overexpression in the muscle (Awede et al., 2002). This demonstrated the effect of GH and IGF-1 are not to specifically induce muscle hypertrophy, and there are potentially different pathways by which GH can affect muscle hypertrophy, which results in the differences observed between *in vivo* and *in vitro*.

4.4.2 Effects of metabolic modifiers on lipid profile of sheep subcutaneous fat and *suprasupinatus* muscle tissue

The BA and GH have been widely described as the repartitioning agents, which redirect nutrient away from subcutaneous fat and towards other tissues such as skeletal muscles. As indicated in the literature, several studies have demonstrated the effects of BA and GH in reducing lipogenesis and increasing lipolysis (Chaves et al., 2013; Lynch and Ryall, 2008; Wang et al., 1999; Yang and McElligott, 1989; Smith, 1987; Duquette and Muir, 1985), which lead to the reduced lipid deposition (Dunshea et al., 2005). Johnson et al. (2014) reported that BA reduced lipogenesis, and stimulated lipolysis in adipose tissue, suggesting BA directly affect total lipid in the tissue. GH treatment also has been reported to reduce lipid accretion in the muscle (Smith, 1991).

To the best of our knowledge, this was the first time the analysis of fatty acid profile upon the metabolic modifiers treatment has been done. Only BA treatment reduced the palmitic acid (C16:0) concentration in the subcutaneous adipose tissue samples, while the fatty acid composition in *suprasupinatus* muscle samples were unaffected by both treatments. It has been reported that the repartitioning agents should decrease the saturated fats, which are categorized as storage lipids (Smith, 1991). As the reduction in palmitic acid proportion, the unsaturated/saturated fatty acid ratio tended to increase, indicated that the unsaturated fatty acid proportion in the subcutaneous adipose tissue samples appeared to increase after BA treatment, as similar with Hu et al. (1988). This effect of BA might influence the meat quality criteria such as firmness (due to melting point of the fatty acids), flavour, and shelf stability (lipid and pigment oxidation) (Wood et al., 2008). However, the effect pattern of metabolic modifiers on fatty acid composition was not consistent. The effect of BA treatment induced a higher proportion of oleic acid (C18:0) in subcutaneous fat and in steers, while shifted towards saturated fatty acids in the *longissimus dorsi* muscle (Webb and O'Neill, 2008).

4.4.3 Effects of metabolic modifiers on sheep metabolomics analysis

In current study, BA and GH induced the glycerol and monoacylglycerol concentration in plasma, while the circulating NEFAs were generally elevated by the GH treatment, indicating the induction of lipolysis after both treatments, but indicating stronger effects in GH-treated animals. Study by O'Connor et al. (1991) demonstrated the increase of plasma glycerol and NEFAs in lambs as early as within 2 hours after treatment. The similar observations were seen after BA treatment in cattle (Eisemann et al., 1988), which was demonstrated to be mediated by β-ARs (Blum et al., 1982), and adipose tissue explants (increase release of glycerol and NEFAs into the media) (Peterla and Scanes, 1990). This lipolytic effect thought to be mediated by the action of HSL via the cAMP/PKA pathway via β-ARs stimulation (Chaves et al., 2013; Yang et al., 2004; Lonnqvist et al., 1993). The lipolytic action of GH on adipose tissue by increasing circulating NEFA and glycerol concentration have been reported in rats, human, pigs, and adipocytes such as 3T3-F442A cells, which is thought to be mediated by the action of HSL via the cAMP/PKA pathway, which is more pronounced in the visceral compared to subcutaneous depots (Chaves et al., 2013; Wang et al., 1999; Wabitsch et al., 1996; Lonnqvist et al., 1993; Dietz and Schwartz, 1991). As the adipose tissue is the major reservoir of cholesterol, and therefore the increase plasma cholesterol possibly reflects a cholesterol efflux to high density lipoprotein (HDL) in the circulation, thereby indicating the effect of lipolysis in adipose tissue, induced by both BA and GH treatment (Verghese et al., 2007).

Circulating 3-hydroxybutyrate was elevated by the GH treatment indicating increased production of ketone bodies. The ketogenesis (production of ketone bodies) takes place in the liver, which involves transforming NEFAs (by lipid mobilization from adipose tissue) into ketone bodies, before being transported to the peripheral tissues via blood for alternative fuels, such as during fasting (Ferrier, 2014). This also suggested that the NEFAs concentration influenced the rate of ketogenesis, and thus suggested the GH treatment is involved in fatty acid oxidation or lipolysis (Møller and Jørgensen., 2009; Start and Newsholme, 1968). The NEFAs and ketone bodies (such as 3-hydroxybutyrate) have been

considered as biomarkers for negative energy balance (associated with increase tissue mobilization) in cows (Ospina et al., 2013; Taylor et al., 2003). As the lambs were not fasted in current study, the increase in NEFAs, by GH treatment might have led to development of oxidative stress by the production of oxygen radicals, such as reactive oxygen species (ROS). An increase oxidative stress is associated with insulin resistance which might have started to develop in these animals, although no measure on insulin was made in these studies (Youssef and El-Ashker, 2017; Xu et al., 2014).

4.4.3.1 Effects of metabolic modifiers on plasma glucose and non-carbohydrate carbon metabolites

Glycolysis is a process of conversion of glucose into pyruvates (aerobic) and lactate (anaerobic). Current findings demonstrated GH treatment elevated plasma glucose and pyruvates, whilst plasma lactate tended to increase with the BA treatment. The high plasma glucose level in GH treated sheep implied that there was reduced insulin sensitivity on the tissues such as liver, fat and muscles resulting in a reduced clearance of glucose. However, neither insulin nor glucose tolerance or insulin sensitivity were measured in this trial. The increase in glucose was associated with increased availability and delivery of glycerol (from lipolysis) to the liver, as a substrate to promote gluconeogenesis.

Earlier interpretations of GH effects suggested that it was involved in several pathways in regulating glucose homeostasis by (a) stimulating uptake and incorporation of amino acids into protein, (b) stimulating fatty acids oxidation and mobilization from adipose tissue, and (c) antagonizing the effects of insulin and increasing the concentration of plasma glucose, which all these contribute to the consideration of the GH as ‘lipolytic’ and ‘diabetogenic’ (McDowell, 1983). It has previously been demonstrated that exogenous GH increased plasma glucose concentration in lactating cows, which was accompanied with increased NEFAs, and increased its uptake into mammary tissues, suggesting GH regulates the supply and utilization the key metabolites, especially on mammary tissues (McDowell et al., 1987). In addition, the gluconeogenesis from propionate was increased in the liver explanted from GH-injected cows (Pocius and Herbein,

1986). Meanwhile, chronic injection of porcine GH on both fed and fasted pigs increased plasma glucose, as well as insulin concentration, with no effect on acute treatment (Gopinath and Etherton, 1989). In GH-treated cow, the increase in plasma glucose might suggest the increase in insulin resistance (Dominici et al., 1999). This lead to the suggestion that there was development of insulin resistance in GH-treated lambs in current study.

Several studies also have demonstrated the effect of BA on regulating blood glucose and insulin sensitivity with the consequence of affecting the utilization of metabolites in peripheral tissues. In humans, the high dose of isoproterenol (non-selective BA) increase plasma glucose, insulin and NEFAs, suggested the involvement of β-ARs (Porte, 1967). This was confirmed by the studies of the effects of terbutaline (selective BA) in rats and several adrenergic blocking drugs in rabbits where there were similar effects as seen in humans on those metabolites, which led to the conclusion that the effects were mediated via β_2-receptors (Knudtzon, 1984; Ahrén et al., 1981). In ducks, the effects of β-blocker (propanolol) in reducing in plasma insulin and increase NEFAs concentration also suggested the above conclusion (Gross and Mialhe, 1982). Meanwhile, the availability of NEFAs induced the competition with glucose for oxidative substrate, and could result in reduced oxidation and utilization of glucose (Møller and Jørgensen, 2009; Russell-Jones and Umpleby, 1999), as well as impaired insulin action in target tissues (Vijayakumar et al., 2010). The lipolytic effect and increase insulin resistance of GH, were associated with increase of pyruvate dehydrogenase kinase-4 (PDK4) mRNA expression and reduced pyruvate dehydrogenase complex (PDC) activity, resulted in inhibition of pyruvate conversion to acetyl-CoA, which might have suggested the elevated plasma pyruvate concentration (Zhang et al., 2014; Jeong et al., 2012).

The adrenaline (a ligand for β_2-receptors) infusion increased plasma glucose and reduced glucose utilization, whilst increasing insulin resistance. This was probably due to glucagon stimulation and increased glycogenolysis and gluconeogenesis, but only for few hours before gradually decreasing (McDowell, 1983). The acute (6-12 hours) BA treatment induced the increase of both plasma glucose and insulin concentration in lambs, but chronic treatment

(3 weeks and 6 weeks) reduced insulin level, with no change in glucose level (O'Connor et al., 1991). BA also temporarily increase plasma glucose, insulin and NEFAs within 6 hours (Blum and Flueckiger, 1988). Thus, in present study, we suggested the insignificant effects on the glucose level by BA treatment was due changes over time, the concentration probably being high in the initial treatment hours and gradually reducing after few days onwards.

There was also a trend of the BA treatment in increasing plasma lactate concentration. In human, the plasma lactate, as well as glucose and NEFAs concentration were increased in adrenaline treatment of humans, without any effect on insulin level (Ensinger et al., 1992). Presumably this is due to increased glycolysis, resulting in increased pyruvate production (Gore et al., 1996). In animals, the BA also induced plasma lactate production in rats (Li and Jefferson, 1977), pigs (Warriss et al., 1990), and lambs (Warriss et al., 1989). Vestergaard et al. (1994) reported that the stimulation of anaerobic glycolytic capacity by BA treatment was associated with the increase of lactate dehydrogenase activity. The circulating lactate was likely increased in BA treated-lambs by anaerobic metabolism, which might due to increase glycogenolysis in LD muscle (Haffner and Kendall, 1992).

4.4.3.2 Effects of metabolic modifiers on plasma glucogenic and ketogenic amino acid concentration

The amino acids are involved in the production of glucose (glucogenic amino acids) and ketone bodies (ketogenic amino acids) or both. The measurement of the circulating amino acids concentration indicates the metabolic condition of the animal after treatment with BA and GH.

Generally, in the current study, the plasma concentration of some individual amino acids were decreased after treatment with metabolic modifiers, with GH showed stronger effect than BA. The GH treatment reduced the plasma concentration of glucogenic non-essential amino acids such as glutamine and glycine as well as tending in reducing serine and proline, and the essential amino acid, methionine. Meanwhile, BA treatment generally reduced plasma

concentration of glucogenic essential amino acids such as histidine and methionine. Therefore, this suggests that both treatments, particularly GH, were important in promoting the amino acids mobilization for the utilization for glucose production in liver. To support this, it has been reported earlier that the plasma, as well as in the liver glucogenic amino acids concentration were reduced in diabetic rats, which is consistent with our suggestion toward GH-inducing insulin resistance, presumably indicating the liver utilization for the glucose production, demonstrated by increased incorporation of labelled amino acids into glucose (Mallette et al., 1969; Scharff and Wool, 1966).

When considering the essential amino acids, the reduction of circulating histidine and methionine by BA treatment, might suggested the utilization of those amino acids for the muscle hypertrophy. In addition, the regulation of skeletal muscle protein metabolism was reportedly only to be mediated by the branched chain amino acids (BCAAs), valine, leucine, and isoleucine, as well as three other amino acids (aspartate, asparagine and glutamate) (Norton and Layman, 2006). In this study, the plasma concentration of all these amino acids were not significantly affected by either treatment. However, the BCAAs were numerically reduced by the BA treatment, although not statistically significant. This effect of BA might contribute to the regulation of muscle protein metabolism (Zhang et al., 2011), presumably being used for the anabolism of muscle tissue. Plasma levels of BCAAs also have been used as a marker to predict several metabolic related diseases such as diabetes, obesity and insulin resistance (Ruiz-Canela et al., 2016), due to their suggested role in gluconeogenesis. The increased level of BCAAs have been associated with metabolic syndrome and cardiovascular disease (Lynch and Adams, 2014). The increased plasma level of the BCAA, leucine, which is the main activator for protein synthesis via mTOR (Norton and Layman, 2006) is believed to be involved in uncoupling of insulin signalling, which contributed to insulin resistance associated obesity (Lynch and Adams, 2014). Insulin resistance was found to be associated with a reduction of branched-chain alpha-keto acid dehydrogenase complex activity which suppresses BCAAs catabolism (Bi and Henry, 2017). This is presumably involved in the increased level of amino acids, as well as the increased glucose level in GH-treated lambs.

The degradation/breakdown of amino acids, including BCAAs also can be determined by their circulating catabolic metabolites. The alpha-hydroxyisocaproate (HICA) is an end product of leucine catabolism (Mero et al., 2010). The induction of plasma level of HICA by GH treatment, suggesting the leucine catabolism was increased by GH, and increased metabolism of leucine, toward ketogenesis, as it categorized as ketogenic amino acid. Similar effect of GH was observed in inducing 2-hydroxybutyrate level, which is the metabolite for methionine and threonine catabolism. This metabolite was thought to positively correlate with increased NEFAs (Gall et al., 2010), suggesting increase lipid oxidation by GH, which potential leads to increased insulin resistance in GH-treated lambs as described earlier. The metabolite plasma concentration of uroconate (known uroconic acid), which is the intermediate in histidine degradation for α-ketoglutarate production, as well as indoleacetate, which is the product from tryptophan catabolism, were significantly reduced by GH. This presumably suggests that degradation of both amino acids were lower in GH treatment, and more amino acids were potentially used for the glucose and ketone bodies production. The 'diabetogenic' effect of GH also was confirmed by the elevation of plasma beta-hydroxypyruvate concentration, as it reported to associate with increased in plasma glucose in mice and with type II diabetes in human (Mizuno, 2015; McDowell, 1983).

4.4.4 Effects of metabolic modifiers on lipogenic and lipolytic gene expression

The lipid metabolism enzymes Acetyl-CoA Carboxylase (ACC) and Fatty Acid Synthase (FAS), involved in lipogenesis, and Adipose Triglyceride Lipase (ATGL) and Hormone Sensitive Lipase (HSL), involved in lipolysis, have a rate-limiting role in the accumulation and metabolism of lipid, as they control the fatty acids synthesization and hydrolyzation, respectively (Etherton and Bauman, 1998). Alterations in the activity of these enzymes are associated with the changes in the rate of lipid metabolism, as these enzymes are responsible for rate of *de novo* lipogenesis and lipolysis (José et al., 2006). It has been demonstrated in several studies BA and GH treatments influence lipid metabolism both reducing lipogenesis (Mersmann, 2002; Etherton et al., 1993;

Yang and McElligott, 1989) and inducing lipolysis (Chaves et al., 2013; Louis et al., 2000; Lonnqvist et al., 1993), however the mechanisms of this influence have not been full elucidated particularly the examination at the level of gene expression, especially simultaneously across tissues that are involved in their metabolism and the response to the metabolic modifiers, skeletal muscles, liver and adipose tissue.

In current studies, the mRNA expression of ACC was tended to be lower in subcutaneous adipose tissue in GH-treated lambs, whilst being unaffected in other tissue. There was significantly reduced FAS mRNA expression in subcutaneous fat, suggesting the stronger effect of GH than BA in reducing lipogenesis in adipose tissue. An indication of this effect was reported by Goodman. (1963) in rats, where there was reduction of extractable lipid from adipose tissue after GH treatment. Molecular studies demonstrated the reduction of ACC mRNA activity after GH treatment in adipose tissue in pigs, lactating cows and cultured adipose tissue from sheep (Lanna et al., 1995; Liu et al., 1994; Vernon et al., 1991). Harris et al. (1993) also reported the reduction of FAS activity in adipose tissue of growing pigs after somatotropin treatment. The *in vitro* study by José et al. (2006), demonstrated the reduction of FAS activity in adipose tissue explants after 48 hours treatment with porcine GH (pGH). In addition, the reduction in lipid synthesis rates might associated with the glucose (in pigs) and lactate (in ruminants) incorporation (José et al., 2006). Apart of FAS, ACC was also demonstrated in several studies to be decreased by the effect of GH treatment, both in ruminants, such as sheep (Vernon et al., 1991) and cows (Lanna and Bauman, 1999), and non-ruminants, pigs (Donkin et al., 1996; Lanna et al., 1995; Harris et al., 1993) as well as isolated adipocytes (Yin et al., 1998; Magri et al., 1990). Meanwhile, the study by Beswick and Kennelly. (1998) demonstrated the increase of both genes, ACC and FAS, in adipose tissue in GH-treated cow, whilst there was no effect in mammary glands. This suggested the nutrient redirection from adipose tissue to the mammary tissue for the milk production.

The BA treatment did not affect any changes in the lipogenic genes in subcutaneous adipose tissue. This finding is supported by an earlier *in vitro* study

by Mersmann. (1989), which demonstrated there was no anti-lipogenic effect by BA on porcine adipose tissue. However, several studies demonstrated the effect BA in reducing lipogenesis, by inhibiting key lipogenic enzymes, which leads to the decreased in adipose tissue hypertrophy (Mersmann, 2002; Yang and McElligott, 1989). The FAS expression, as well as other lipogenic genes such as SREBP-1 were reduced in adipose tissue of ractopamine-treated finishing pigs (Halsey et al., 2011; Reiter et al., 2007). Thus, in this study, the GH treatment showed stronger anti-lipogenic capacity in adipose tissue compared to BA.

The effect of metabolic modifiers on lipogenesis in the muscles is unclear, as the previous studies have focused more on the lipogenesis in the adipose tissue. In current study, the GH treatment reduced FAS, not ACC expression in both muscles, SS and LD, whilst the BA influenced a significant downregulation of FAS expression only in SS muscle. This suggests that both treatments have effects in promoting lean muscle development by reducing lipid synthetic capacity in the muscles. This was in line with the activation of FAS and ACC which were reflected to the rate of marbling in muscle. Qiao et al. (2007) suggested that FAS may function as an enzyme of marbling storage and has diverse influence in different species. They also suggested that adipose tissue at different growth periods may have different activity of lipid turnover related enzymes, which could cause the expression pattern differences between the tissues. Increase in expression of FAS and ACC is associated with the increased marbling (Ward et al., 2010; Underwood et al., 2008). The reduction in marbling score was shown in the muscle of the BA-treated steers (Schiavetta et al., 1990), resulted in reduced quality of meat.

The FAS expression reduced by both treatments might be indicative of insulin resistance (Funai et al., 2013). This effect has not been extensively studied. However, an indication of insulin resistance in current study is suggested by the GH-induced plasma glucose. Insulin has been reported to regulate the activation of lipogenic genes and the glucose affects the stimulation of FAS and ACC transcriptions (Ladeira et al., 2016). GH also had the effect of reducing the mRNA expression of glucose transporter-1 (GLUT-1) in adipose tissue, resulting in reduced insulin sensitivity and reduced glucose uptake in adipose

tissue (Vijayakumar et al., 2010). This effect was also indicated by the GH-reduced adiponectin mRNA expression in adipose tissue, which correlated with increase insulin resistance (Kadowaki et al., 2008). This may explain the increase in plasma glucose level after GH treatment, suggesting GH antagonized the action of insulin. In addition, the reduction of glucose incorporation into fatty acids by GH treatment has also been reported as being involved in the reduction in lipogenesis (Wang et al., 1999), which is reflected in the downregulation of FAS and ACC expression in the adipose tissue as well as the increased glucose plasma concentration.

In regard to the lipolysis BA tended to reduce lipolytic genes (HSL and ATGL) in SS muscle, suggesting the effect of BA in the reduction of lipid turnover in muscle. This also suggested lipolysis in muscle of the treated sheep was lower than non-treated animals. This disagrees with the expectation that GH and BA would increase the expression of lipolytic genes, which will mobilise fat from muscle and produce lean meat (lower fat content). However, according to Kazala et al. (2003), in muscle tissue, triacylglycerol is degraded by the combined action of HSL, diacylglycerol lipase and monoacylglycerol lipase, but hydrolytic activity of HSL is considerably lower than the activities of the two other lipases. Meanwhile, both treatments did not appear to induce lipolysis in the subcutaneous adipose tissue. It has been described by Vijayakumar et al. (2010), that the lipolytic effect of GH was predominantly on visceral adipose tissue, which was not quantified in this study, to a lesser extent in subcutaneous adipose tissue. Therefore, the increase in plasma NEFAs and glycerol after GH treatment in the current study, might due to the lipolysis or fat mobilization from the visceral fats.

The systematic review by Ferreira et al. (2013) reported the effects of BA in reducing lipid accretion may be more related in reducing lipogenesis than inducing lipolysis. BA also has been demonstrated to reduce insulin sensitivity in adipocytes, which leads to reduction in lipogenesis (Mills and Liu, 1990; Mills et al., 1990). The ATGL gene expression in mammals (humans, mice, pigs and cattle) appears predominantly to be in adipose tissue, with much lower expression in muscle tissues which seems to indicate that the gene was related

to fat deposition and carcass traits (Xiaomei et al., 2012). The other enzyme involved in lipid metabolism is HSL which plays a pivotal role in providing the major source of energy for most tissues. Although its expression is highest in adipose tissue, HSL is also expressed in adrenal, ovary, testis, and to a lesser extent in skeletal and cardiac muscle and macrophages (Kraemer and Shen, 2002).

Generally, regulation of gene expression of the key enzymes of lipid metabolism is also controlled by several factors including animal age, nutritional state, diet composition, proportion of carbohydrates and fats, profile and degree of saturation of the fatty acids as well as hormonal effects (José, et al., 2006). According to Fennessy et al. (1990), the effects of BA such as cimaterol are dose dependent in sheep. Their comparison for 12 weeks treatment between two dosages of cimaterol in lambs resulted in a greater reduction in fat even at lower dosage rate, while the 6 weeks treatment only reduce the fat deposition with higher dosage. This means that the length of the treatment might also influenced the fat deposition, which could be affected the results in the gene expression levels in this experiment.

However, it must be remembered that enzyme activities are predominantly determined by the extensive posttranscriptional or posttranslational regulation, whilst mRNA expression is often not a completely definitive indicator of enzyme activity. The induction of fatty acids and glycerol release from fat cells in response to BA stimulation is not associated with increased levels of either ATGL or HSL mRNA. In fact, in some studies, isoproterenol treatment of 3T3-L1 cells or isolated adipocytes causes decreased mRNA concentrations of both lipases. This suggests that the BA stimulation of lipolysis is predominantly regulated post-transcriptionally (Zechner et al., 2009). In addition, the GH stimulation on lipolysis was due to the increased lipolytic gene activities, with an unclear effect on mRNA expression (Vijayakumar et al., 2010). Thus, the quantification of the lipolysis could be improved, by assessing not only on mRNA expression but also determining protein quantities and assessing other fat depots such as visceral fat. Furthermore, the inconsistent findings on increase NEFAs and glycerol level, with reduce in lipase mRNA expression, suggested

that the mRNA quantification was not sufficient for the determination of enzyme activities, and have to consider posttranscriptional or posttranslational regulation (Zechner et al., 2009).

4.5 CONCLUSION

The GH treatment showed a strong effect in the lipid metabolism compared to BA, which more pronounced in skeletal muscle hypertrophy. In the mRNA level, GH was demonstrated to reduce lipogenesis rather than lipolysis, promoting for lean tissue development particularly in the muscle. Therefore, the increase in circulating NEFAs and glycerol level in the plasma might be due to the visceral fat lipolysis. The increase plasma NEFA concentrations could potentially lead to an increased insulin resistance. Indeed, this situation was indicated by an increase in plasma glucose and the mobilization of glucogenic amino acids. Part of the GH treatment effect therefore is the potential inhibition of glucose uptake in the peripheral tissues, which in turn could reduce lipogenic gene expression.

5. EFFECTS OF METABOLIC MODIFIERS ON LIPID METABOLISM IN MYOBLAST C2C12 CELL LINES

5.1 INTRODUCTION

Over sixty percent of human dietary intake is derived from the animal products. As the growing world population becomes critical, the efficiency in food production is required to accommodate the food demand. As meat becomes more popular, there is a concern about meat quality. One of the determinants of meat quality is the quantity of intramuscular fat (IMF). Therefore, the investigation of IMF deposition has been the main target of researchers involved with animal growth. This area is also of interest to human health, as the understanding of the mechanisms of IMF deposition has implications for the metabolic function of muscle, but also because of a dietary consideration as the consumption of meat containing large quantities of fat has implications for nutritionally related health problems, such cardiovascular disease.

Due the considerations for human health which affected by dietary intake, because of the presence of fat and high risk of cardiovascular disease more studies have been conducted to reduce the risk and producing lean meat rather than focusing on the palatability of the meat, which is influenced by the quantity of IMF. Previous research has focused on the identification of metabolic modifiers/growth promoters which increase the efficiency in animal production. These agents redirect nutrients, increasing protein synthesis for muscle development and reducing lipid synthesis, which can give leaner carcasses. Beta-agonists (BAs) have strong effects in the muscle hypertrophy as they increase skeletal muscle mass and/or cross-sectional area of muscle fibers, including the increasing responsiveness of the type II fibres. They do this by the activating beta-agonist receptors, which in turn activates cyclic adenosine monophosphate (cAMP) signalling pathway in the muscle (Johnson et al., 2014). Generally they increase expression of fast myosin heavy chain isoforms ultimetaly resulting in hypertrophy the type II fibres (Johnson et al., 2014). Growth hormone (GH) also

influences the growth of the muscles and adipose tissue (Dikeman and Devine, 2014).

Apart from affecting myogenesis *in vitro* (van der Meijden et al., 2016; Girgis et al., 2014), a recent study by Chang and Kim. (2016) showed that the active form of vitamin D, calcitriol (1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$]), affects the fat storage in 3T3-L1 cells by inducing lipolysis. However, Ryan et al. (2013) suggested that dose-dependent of $1,25(OH)_2D_3$ induced the transdifferentiation of myoblast to adipocytes in C2C12 cultured cells, and inhibition of fat accumulation by high concentration of $1,25(OH)_2D_3$. These studies suggested the potent effects of $1,25(OH)_2D_3$ in lipid metabolism particularly adipogenesis in muscle cells in culture. Therefore, this study sort to utilise this effect of $1,25(OH)_2D_3$ to generate a cell culture system which was co-cultured of muscle cells with adipocytes. Following this preincubation to generate a mixed cell population, we could then investiagte the effects of metabolic modifiers.

While BA and GH exert the effect on the protein and lipid metabolism *in vivo*, their effect on the lipid metabolism particularly the potential interactions between adipocytes and muscle cells in muscle tissue is not clear. Most of the studies only measured their effect on C2C12 cell lines in terms of myogenesis (Wannenes et al., 2012; Imanaka et al., 2008). Thus, taken together, we aim to elucidate the effect of the BA and GH on the lipid metabolism in terms of the adipogenesis, lipogenesis, and lipolytic effects *in vitro* when adipocytes are co-cultured with muscle cells, to investigate the impact of BA and GH on these co-cultured cells. In this experiment, C2C12 were used as a model for the skeletal muscle and transdifferentiated in adipogenic media to induce development of adipocytes containing fat droplets as model of intramuscular fat model. We hypothesized that dibutyryl cyclic adenosine monophosphate (dbcAMP) an analogue of cAMP, which mimics the effects of BA stimulated BARs, and Des (1-3) IGF-1, which mimics the effect of IGF-1 would:

1) affect the expression of the myogenic and lipogenic genes in a similar manner to as seen in the *in vivo* work in the sheep study.
2) inhibit the formation of lipid droplets formation
3) stimulate myotubes development/growth.

5.2 RESULTS

This section included the description of method optimization for the establishment of the transdifferentiated-myogenic cell culture model to exhibit the "true" muscle model, which consists of muscle fibres and intramuscular fat. This work was included to increase the number of lipid droplets by incubation in adipogenic media and $1,25(OH)_2D_3$ treatment whilst at the same time determining the expression of myogenic- and adipogenic-related genes. After establishing this model, the effects of treating with dbcAMP and Des (1-3) IGF-1 were examined. The flow of the research work is as follow:

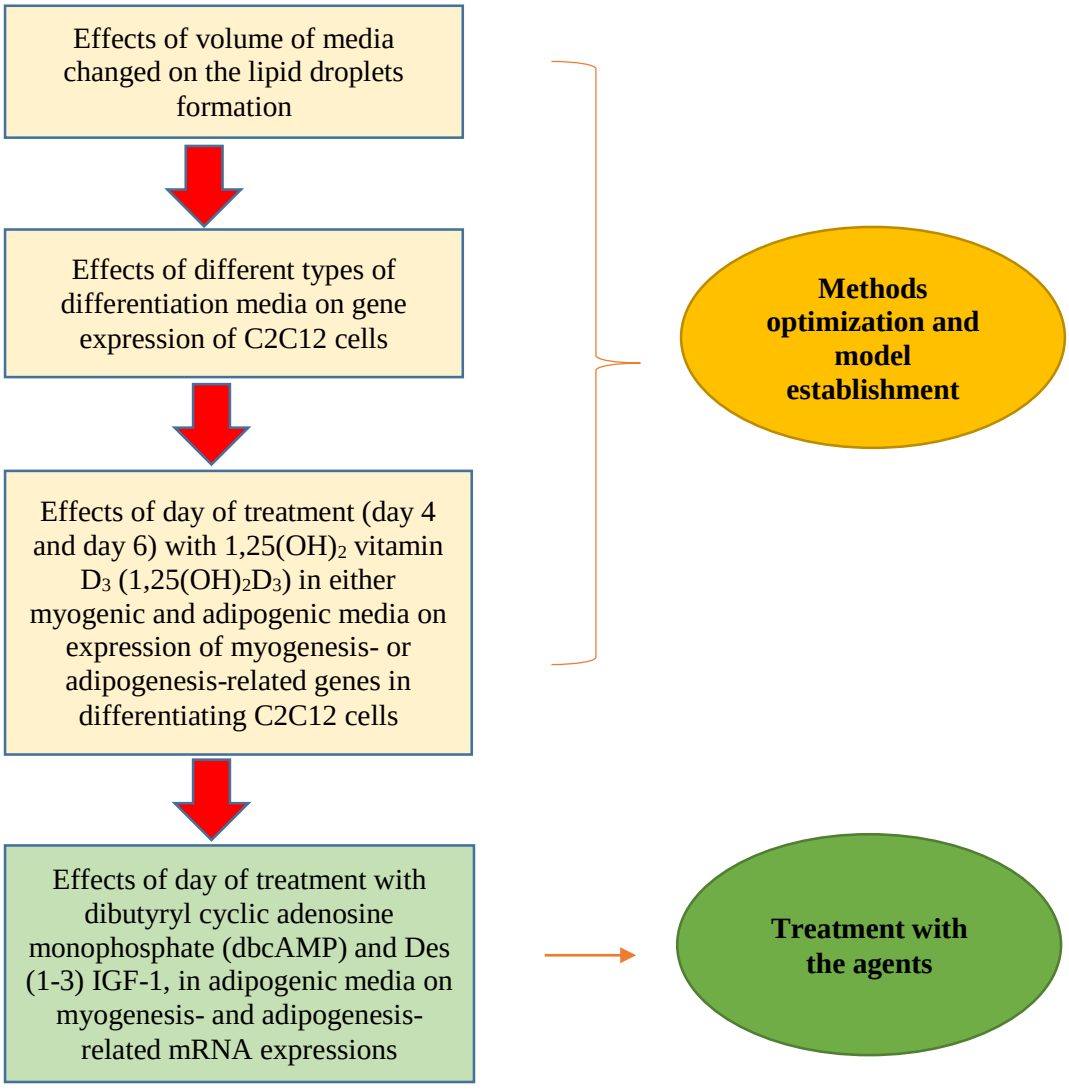


Figure 5.1: Flow of the methods optimization and establishment of the cell culture model to stimulate transdifferentiated-myogenic cells into adipocytes, before treatment with dbcAMP and Des (1-3) IGF-1.

5.2.1 Effects of volume of media changed on the lipid droplets formation

5.2.1.1 Introduction

The growth of the cells in the culture requires an appropriate environment. The cell culture media is enriched with a specific nutrient composition including the glucose, amino acids, and vitamins. During incubation in the media cells rapidly use the nutrient and it becomes depleted quickly. Thus, the nutrient replenishment through media changing plays important roles in order to maintain the healthy cells and allows them to achieve the desired phenotype (Masters and Stacey, 2007). However, the volume of the media to be changed during replenishment is not specified. It is generally recommended all of the media is removed and replaced with fresh at replenishment points during incubation periods. However, there is no specific evidence that indicates the optimal methods for carrying this out. In this experiment, we hypothesized that the amount of the changed media will induce changes in the cell characteristics. We aim to develop an optimization on what volume media should be replaced at replenishment periods to increase the lipid droplets accumulation in the cells treated with adipogenic media.

5.2.1.2 Materials and Methods

The C2C12 myoblast cells were grown in the growth media on 6-well plate, until reached 70-80% confluence. At this point, the media was replaced with adipogenic differentiation media for 6 days. The differentiation media was changed with fresh media for every 2 days. During media changing, the specified volume of media was removed (all, three-quarter, or half of the media volume) (Figure 5.2) and new media used to replace to original volume. After 6 days, the cells were stained with Oil Red-O for the quantification of the lipid droplets accumulation. The detail protocol for the cell culture growth, adipogenic media preparation and adipogenic induction, Oil Red-O staining and lipid droplets quantification were described Section 3.3.

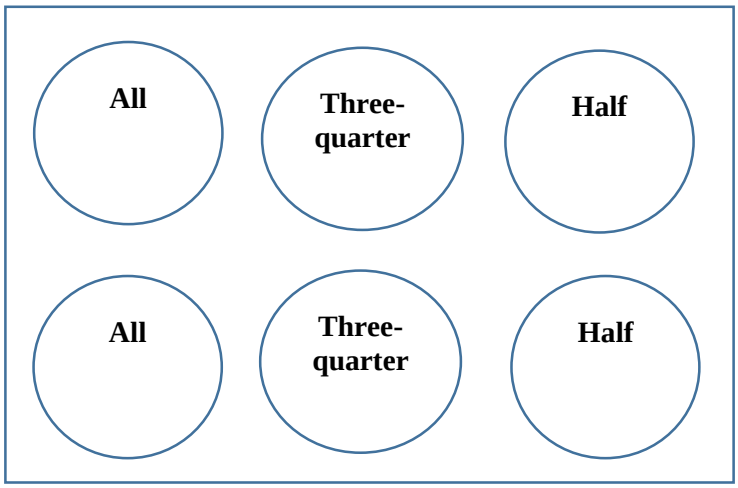


Figure 5.2: The layout of the position regarding the amount of media changes on the 6-well plate. This layout was designed to eliminate the effect of the plate (all factors on one plate). The C2C12 cells were grown and differentiated in duplicated 6-well plates (n=4).

5.2.1.3 Results

According to the representative images after the Oil Red-O staining, it was clearly shown the formation of the lipid droplets in the C2C12 cells in the adipogenic media (Figure 5.3). There was a significant increase in lipid droplets formation ($P<0.01$) in the cells after replenishing half the volume of the media, compared with changing all or three-quarter of the media (in every media changing) (Figure 5.4).

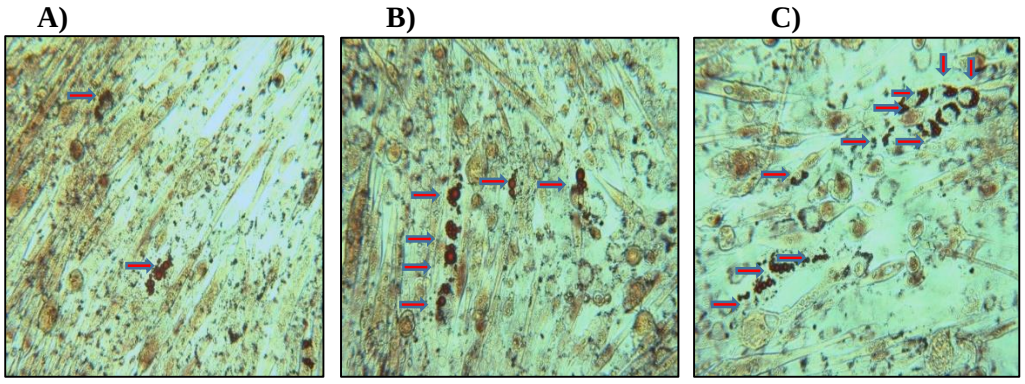


Figure 5.3: Effects of different volume of media changing on the accumulation of lipid droplets. Cells were cultured for six days in adipogenic media with different volume of media changing during incubation period (A (all), B (three-quarter), or C (half)). The lipid droplets (as indicated with the red arrows) were stained with Oil Red-O and visualized under light microscope.

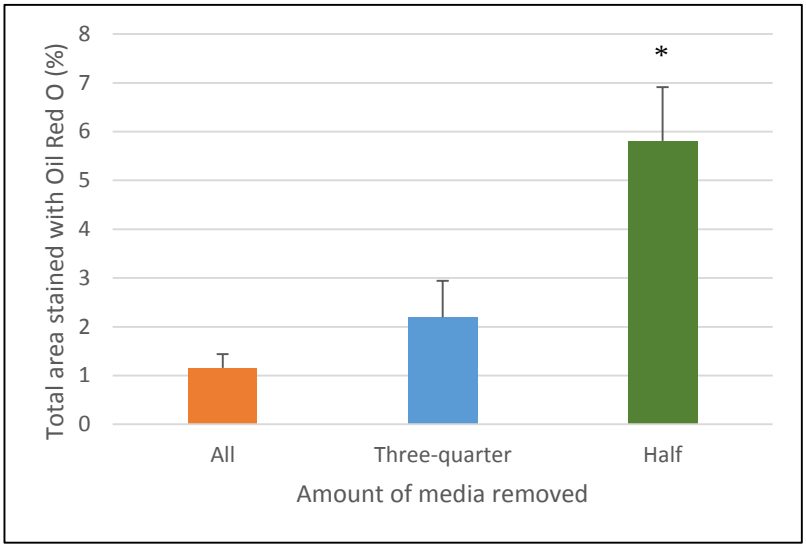


Figure 5.4: The effect of amount of adipogenic media changed, on the percentage area of the lipid droplets in C2C12 cells. The C2C12 cells were exposed to the adipogenic media from the point of 70-80% confluence (day 0) for 6 days with n=4 per group and stained with Oil Red O. The ANOVA was used for statistical comparison between volume removal groups, followed by Bonferroni test. The error bars are ± SEM.
*means significant different at $P<0.05$, among all groups.

5.2.1.4 Summary

In cell culture procedures, media changing is required and important to maintain the cell growth and healthy, by replenish fresh nutrients (Masters and Stacey, 2007). In addition, any changes in media condition also influence the phenotype (morphology) changes to the cells (Masters and Stacey, 2007). In this experiment, the mouse myoblast cell lines, C2C12, have been exposed with adipogenic media, consists of insulin, dexamethasone (DEX) and isobutylmethylxanthine (IBMX), to promote adipogenesis. However, in this study, we just determined the presence of lipid droplets, while no measurement on the presence of adipocytes. Thus, it could be useful to determine the gene expression of adipogenesis markers, to indicate the development of adipogenesis. Previous study demonstrated the induction of adipogenic media had potentially induced the C2C12 transdifferentiation into mature adipocyte, along with the presence of the myotubes formation (Ryan et al., 2013).

The media in cell culture itself contains a complex mixture of different constituents. The basic constituents in the media are glucose, salts and vitamins,

added to these immediately before use are components required to maintain the cell growth (depending on type of cells) which include the serum and antibiotics (Masters and Stacey, 2007). Thus, the media provides an environment which enables a cell to metabolically function. The complex interaction between the cells and the media, and the reaction of cellular signalling, can cause the abundantly released of the autocrine growth factors into the media, which in turn interact with other cells. Thus, in this experiment, the partial media removal and substitution with fresh media in order to replenish the nutrients, also maintained secreted growth factors present in the original media. Presumably, this could be due to the availability of certain metabolites, which already secreted in the media that might induce positive effects on the cell development. These might be important for the cell division and survival, and acts as the substrates for further chemical reaction cascades. Thus, we suggested that removing and replacing half of the media is the best way to optimize the chemical reaction in the cells. Reflected to this finding, in the subsequent cell culture experiments, the differentiation media was changed by half of the original amount during media changing

5.2.2 Effects of different types of differentiation media on gene expression of C2C12 cells

5.2.2.1 Introduction

The objective of this section was to examine the response of the C2C12 myoblast cell line, to different types of differentiation media. Cells were exposed to their normal myogenic differentiation media, which is known to cause myoblasts to fuse and form myotubes, with the associated changes of genes involved in myogenesis and myofibrillar proteins. In comparison, cells were exposed to another set of differentiation media, the adipogenic media, which has been utilised to induce the differentiation of preadipocytes to adipocytes with the associated changes in expression of genes associated with adipocyte cells. Our hypothesis was that the adipogenic media would cause some C2C12 cells to transdifferentiate to adipocytes but there would still be differentiation of C2C12 into myotubes.

5.2.2.2 Materials and Methods

The C2C12 myoblasts were incubated in the normal growth media until reached 70-80% confluency. This day was marked as day 0. The myoblasts were then exposed to either myogenic and adipogenic differentiation media, and then incubated for 6 days, with the media changed with a fresh media for every 2 days. After 6 days treatment, the cells were stained with Oil Red-O for the quantification of the lipid droplets accumulation. The cells were also extracted for the RNA, and evaluated for the gene expression using RT-qPCR. The expression was determined using the standard curve methods, and calibrated either using myogenic or adipogenic pool calibration. In this study, the oligreen concentration was used as the normalisation factor for the RT-qPCR gene expression data as the common reference genes were changed during differentiation (Ryan et al., 2013; Brown et al., 2011). The methods are described in the Section 3.3.

5.2.2.3 Results

5.2.2.3.1 Effects of different types of differentiation media on lipid droplets accumulation

After 6 days incubation, the C2C12 cells in both differentiation media showed visible myotube development. However, Oil Red-O staining confirmed the presence of the lipid droplets in the cells incubated with adipogenic but not myogenic media (Figure 5.5).

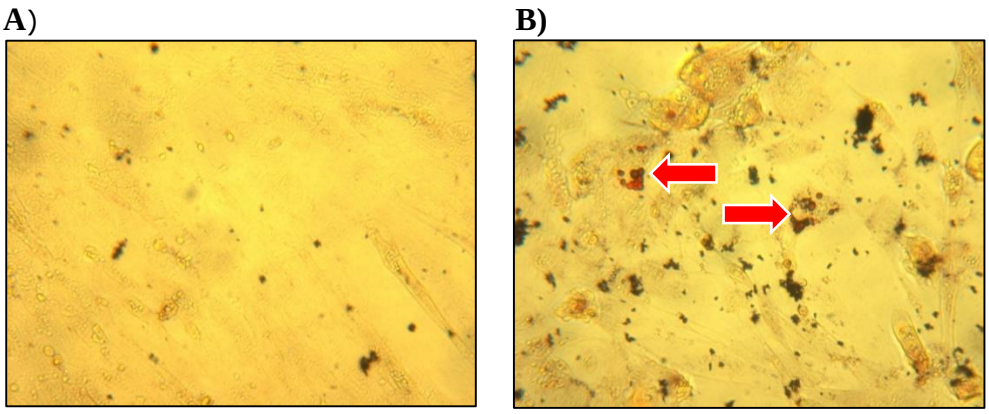


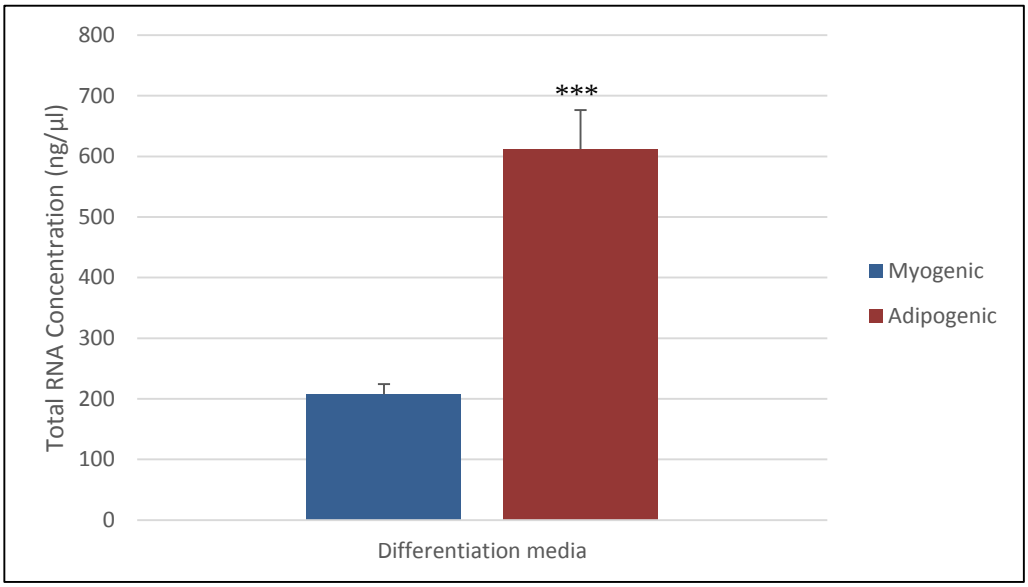
Figure 5.5: The effects of different differentiation media (A) myogenic media and (B) adipogenic media, on the accumulation of lipid droplets. After 6 days incubation in either myogenic or adipogenic media, the C2C12 cells were stained with Oil Red O and the images were visualized using the light microscope. The accumulation of the lipid droplets are indicated by the red arrows.

5.2.2.3.2 Effects of different types of differentiation media on RNA quantity and quality

The total RNA from the C2C12 cells was extracted after 6 days differentiation in myogenic or adipogenic media. There was a highly significant difference ($P<0.001$) in quantity of total RNA concentration extracted from the cells with adipogenic media containing more RNA (Figure 5.6(A)). The absorbance ratio, 260nm/280nm, for the indication of protein contamination, showed no significant difference (Figure 5.6(B)). The ratio reading for the cells were in range (~2.0), which were acceptable as pure and good quality. The quality of the RNAs was also tested by non-denaturing agarose gel electrophoresis (Figure

5.7), which showed two distinct 18S and 28S rRNA bands. This intact rRNA bands was considered a good quality indication for RNA.

A)



B)

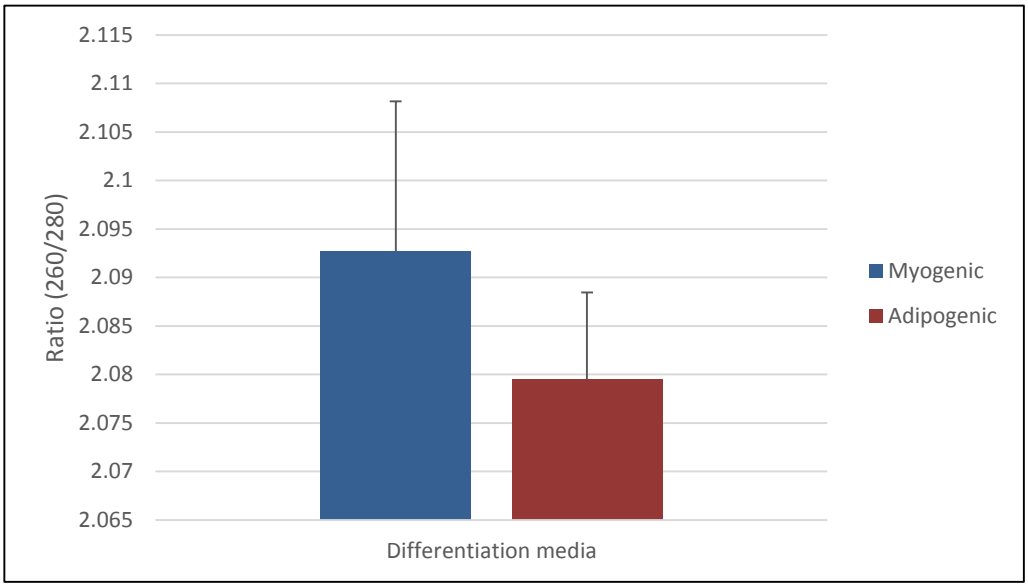


Figure 5.6: Effects of different differentiation media on (A) total RNA concentration and (B) absorbance ratio 260/280 in C2C12 cells. The cells were exposed to either myogenic or adipogenic differentiation media from the point of 70-80% confluence (day 0) for 6 days. The ANOVA was used for statistical comparison. The error bars are ± SEM, n=6 per treatment group.
*** means very highly significant different at P<0.001, by Bonferroni's test

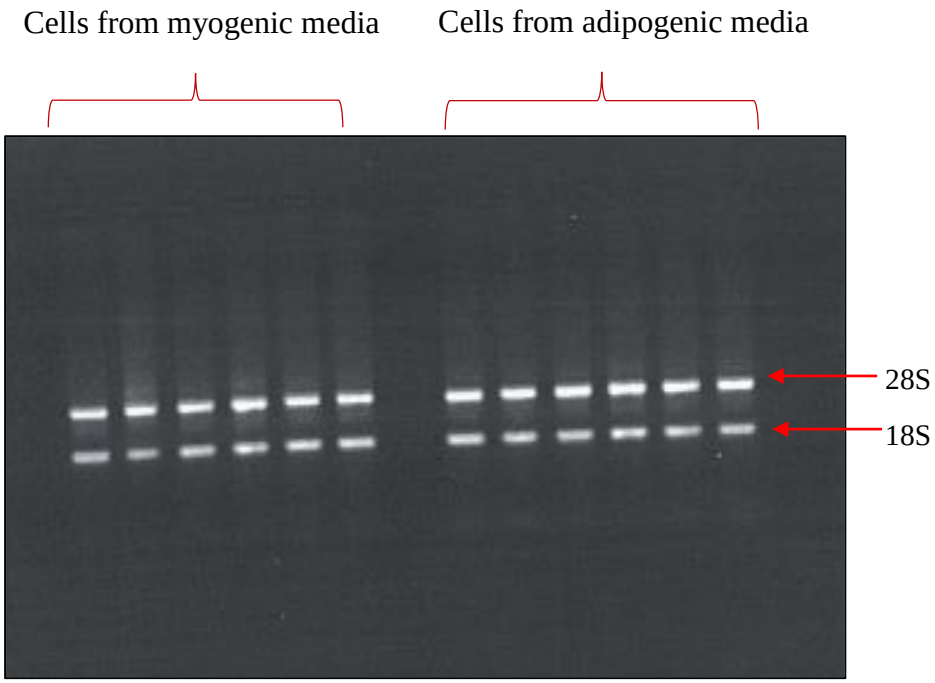


Figure 5.7: Effects of different differentiation media on RNA integrity in C2C12 cells using non-denaturing agarose gel electrophoresis. The cells were exposed to either myogenic or adipogenic differentiation media from the point of 70-80% confluence (day 0) for 6 days. Two distinct bands represent 18S and 28S rRNA.

5.2.2.3.3 Effects of different types of differentiation media on mRNA expression related to myogenesis

In this part, one of the key regulatory factors for myogenesis, myogenin, was evaluated. In addition, creatine kinase (CK) and myosin heavy chain IIa (MyHCIIa) mRNA expressions were evaluated as both are the factors whose expression is an indication of myoblast differentiation to myotubes.

As calibrated by the myogenic pool, there was no significant effect of the oligreen concentration by different type of differentiation media, using the myogenic pool (Figure 5.8). This suggested that the cDNA quantity was not affected by the type of the media. Thus, this concentration was used in the production of the corrected value in the mRNA expression.

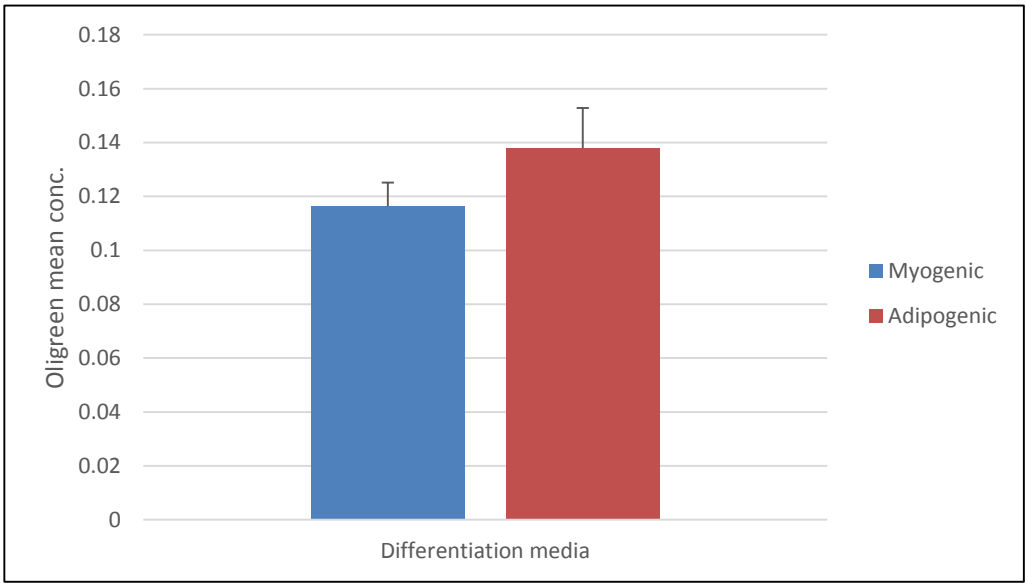
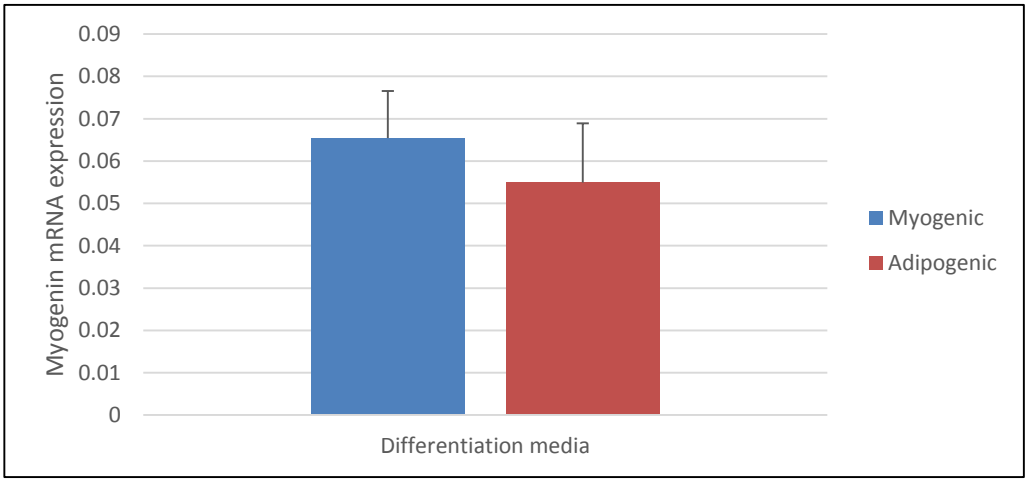


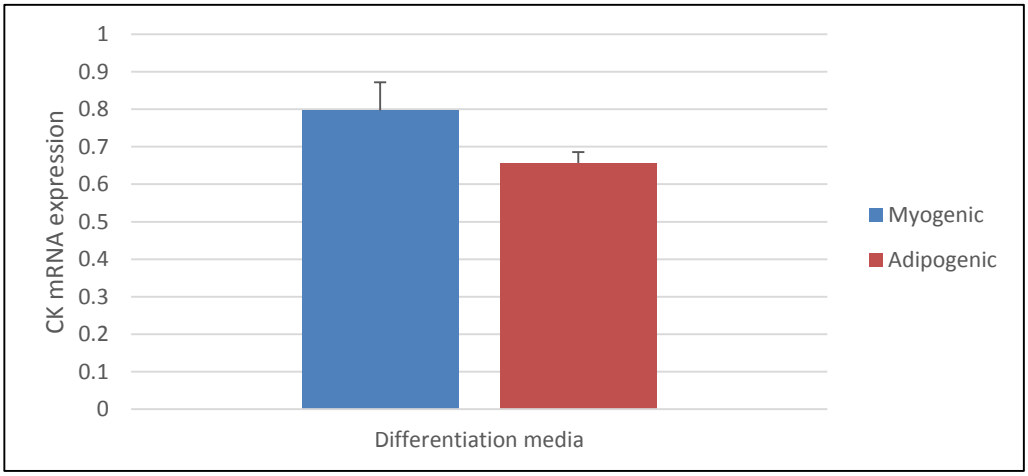
Figure 5.8: Effects of different incubation media on oligreen concentration (corresponding to cDNA quantity) in C2C12 cells, calibrated using myogenic pool. The cells were exposed to either myogenic or adipogenic differentiation media from the point of 70-80% confluence (day 0) for 6 days. The ANOVA was used for statistical comparison. The error bars are ± SEM, n=6 per treatment group.

There were no effects of different type of differentiation media on the myogenin mRNA expression (Figure 5.9(A)). Similarly, there was no significant difference in the expression of the CK mRNA in C2C12 cells incubated with either both media (Figure 5.9(B)). However, there was a significantly higher ($P<0.05$) of the expression of the MyHCIIa mRNA in C2C12 cells incubated in the myogenic media compared to adipogenic media (Figure 5.9(C)).

A)



B)



C)

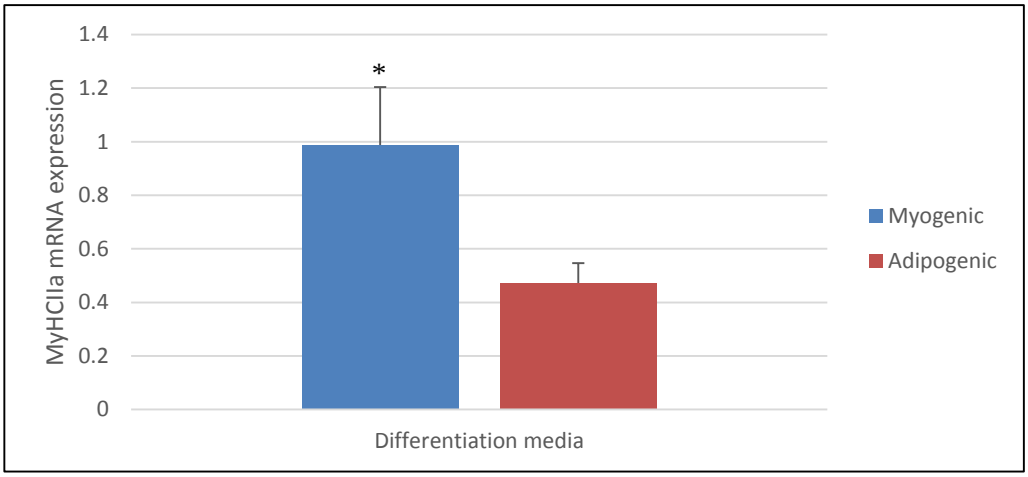


Figure 5.9: Effects of different incubation media on (A) myogenin (B) CK, and (C) MyHCIIa mRNA expressions in C2C12 cells. The cells were exposed to either myogenic or adipogenic differentiation media from the point of 70-80% confluence (day 0) for 6 days. The ANOVA was used for statistical comparison. The error bars are ± SEM, n=6 per treatment group.
* means significant different at $P<0.05$, by Bonferroni's test.

5.2.2.3.4 Effects of different types of differentiation media on mRNA expression related to adipogenesis

Apart of myogenic markers, the effects of the differentiation media on the adipogenic markers mRNA expression were also evaluated. The peroxisome proliferator-activated receptor γ (PPARγ) and fatty acid binding protein-4 (FABP4) are the key adipogenic regulators, which control the adipocyte differentiation, and act as adipogenic markers.

As calibrated using adipogenic pool, the oligreen concentration was not affected by the different differentiation media (Figure 5.10). As above, this oligreen concentration was used to correct the expression value, produced by the adipogenic mRNA expression, for the subsequent statistical analysis.

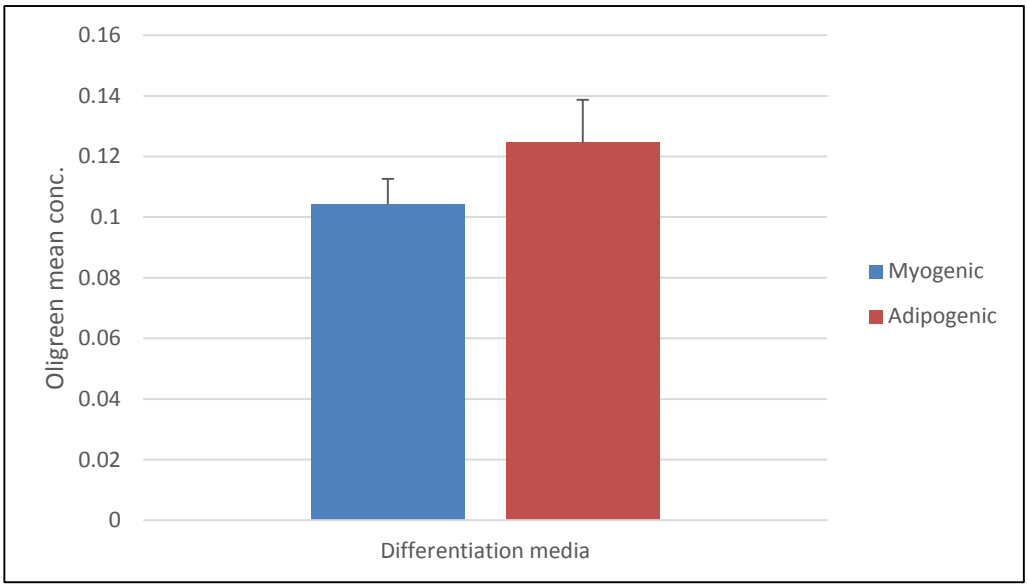
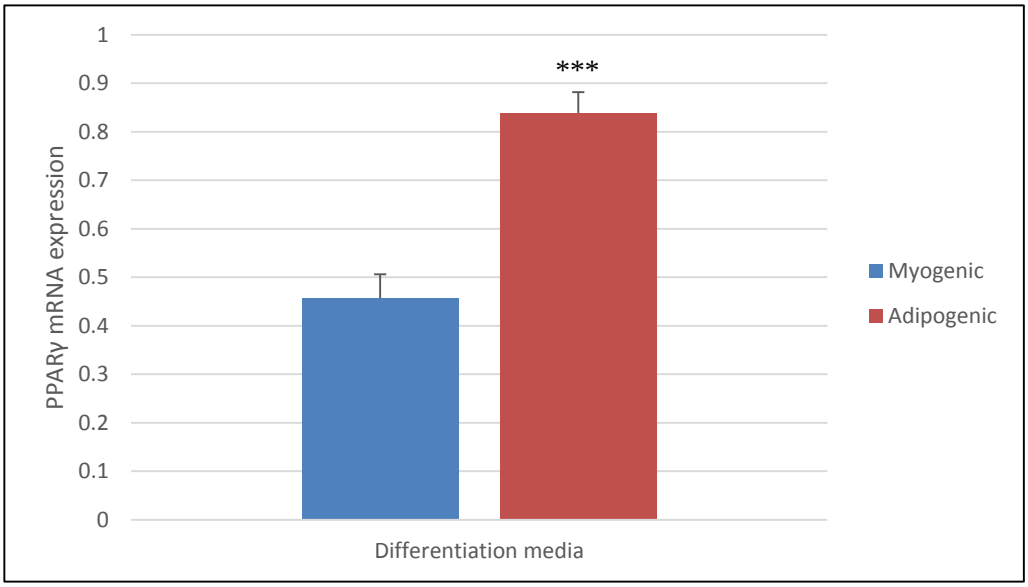


Figure 5.10: Effects of different incubation media on oligreen concentration (corresponding to cDNA quantity) in C2C12 cells, calibrated using adipogenic pool. The cells were exposed to either myogenic or adipogenic differentiation media from the point of 70-80% confluence (day 0) for 6 days. The ANOVA was used for statistical comparison. The error bars are ± SEM, n=6 per treatment group.

After 6 days incubation in different type of differentiation media, there was a very highly significant ($P<0.001$) greater expression of the PPARγ mRNA in C2C12 cells incubated in the adipogenic media compared to myogenic media

(Figure 5.11(A)). The same pattern of expression was also observed in the mRNA expression of FABP4 (P<0.05) (Figure 5.11(B)).

A)



B)

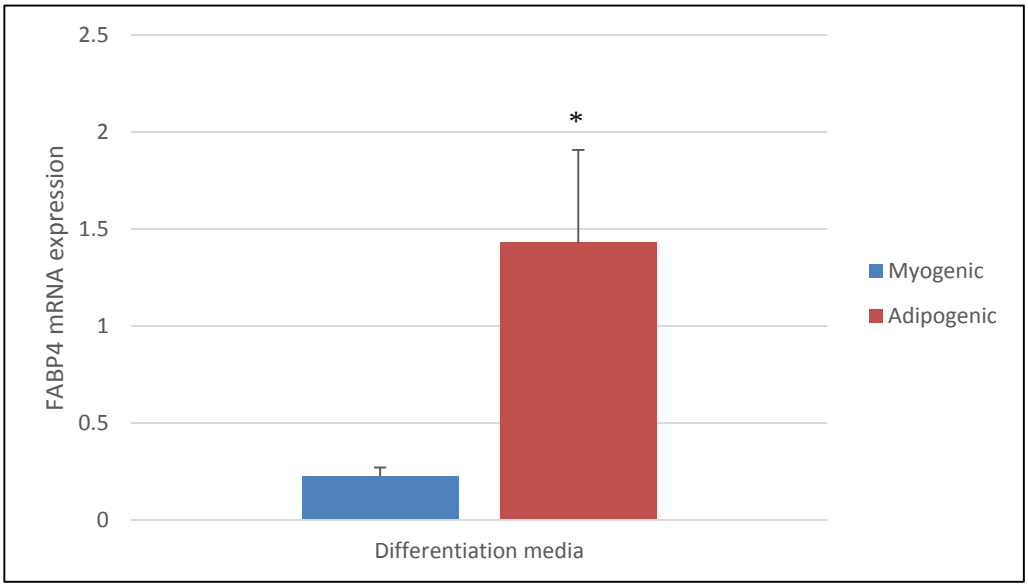


Figure 5.11: Effects of different incubation media on (A) PPARγ, and (B) FABP4 mRNA expressions in C2C12 cells. The cells were exposed to either myogenic or adipogenic differentiation media from the point of 70-80% confluence (day 0) for 6 days. The ANOVA was used for statistical comparison. The error bars are ± SEM, n=6 per treatment group.
* means significant different at P<0.05, by Bonferroni's test.
*** means very highly significant different at P<0.001, by Bonferroni's test.

5.2.2.4 Summary

These findings demonstrated that the myoblast C2C12 cell line could show characteristics of myogenic differentiation as indicated by the expression of mRNA associated with myogenesis. However, in adipogenic media compared to myogenic media, although the mRNA expression of a representative myofibrillar protein and myosin heavy chain, were significantly reduced, other markers of myogenesis were not significantly different between myogenic and adipogenic media. In addition, the incubation of C2C12 cells in the adipogenic differentiation media increased the mRNA expression characteristically associated with the differentiation of adipocytes and adipogenic markers. Thereby demonstrating that the incubation of differentiating C2C12 cells in adipogenic differentiation media does cause adipogenic effects in this myogenic cell line, resulting in gene expression which is characteristic of differentiated adipocytes.

5.2.3 Effects of day of treatment (day 4 and day 6) with 1,25(OH)$_2$ vitamin D$_3$ (1,25(OH)$_2$D$_3$) in either myogenic and adipogenic media on expression of myogenesis- or adipogenesis-related genes in differentiating C2C12 cells.

5.2.3.1 Introduction

It has been demonstrated that vitamin D influences myogenesis in skeletal muscle (Ceglia and Harris, 2013). Several studies suggest the importance of the vitamin D in regulating muscle mass and strength (Tieland et al., 2013; Zamboni et al., 2002). The active form of vitamin D, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) in the serum has been demonstrated to be positively correlated with the musculoskeletal function in young and older individual (Sohl et al., 2013; Ward et al., 2010). An important observation in the context of this is vitamin D deficiency influences the infiltration of fat within the muscle, which is associated muscle weakness and, within the context of human health, the fat infiltration of muscle is associated with insulin resistance (Ryan et al., 2013; Ryall et al., 2008). Ryan et al. (2013) demonstrated myoblast transdifferentiation into adipogenic lineage when incubated with low physiological concentrations of 1,25(OH)$_2$D$_3$, (10^{-13} - 10^{-11} M) in adipogenic media. This was characterised by a significant increase in fat droplet accumulation in C2C12 cells incubated in adipogenic media with 1,25(OH)$_2$D$_3$ when compared to adipogenic media alone. In addition, when 1,25(OH)$_2$D$_3$ was added to adipogenic media there was significantly higher expression of genes associated with adipocytes such as PPARγ and FABP4.

In this section, we aim to investigate whether low physiological concentration of 1,25(OH)$_2$D$_3$ (10^{-10} and 10^{-12} M) affected the proliferation and differentiation in the C2C12 myoblast. We hypothesized that 1,25(OH)$_2$D$_3$ would affect the mRNA expression related to adipogenesis and myogenesis at two different time points of differentiation. This investigation would allow us to choose the optimum concentration of the 1,25(OH)$_2$D$_3$ which could induce fat accumulation in adipocytes in the presence of muscle cells. This potential could then be used as an *in vitro* cell culture based model for intramuscular fat.

5.2.3.2 Materials and Methods

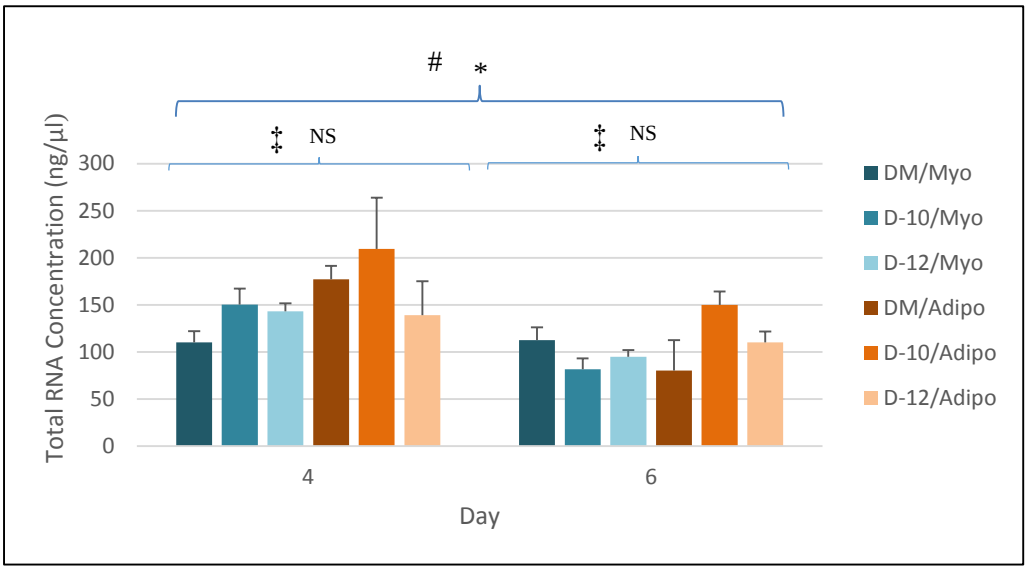
The C2C12 myoblasts were grown and maintained according to the methods described in the Section 3.3. At 70-80% confluency, which marked as day 0, the cells were transferred into either myogenic or adipogenic differentiation media, for 6 days, supplemented with either 0.1% (v/v) dimethyl sulfoxide (DMSO) (as vehicle control), 10^{-10} M (D-10) or 10^{-12} M (D-12) of $1,25(OH)_2D_3$ (Sigma, UK). The cells were stained with Oil Red O staining at day 4 and day 6 of differentiation, and as well as the total RNA were extracted at the same time for the RT-qPCR analysis as described in the Section 3.3.

5.2.3.3 Results

5.2.3.3.1 Dose-dependent effects of treatment either DMSO and $1,25(OH)_2D_3$ (day 4 and day 6) in either myogenic and adipogenic media on RNA quantity and quality.

There was a trend ($P = 0.053$) of increasing the total RNA concentration in adipogenic media compared to myogenic media. There was also a significant difference of total RNA concentration extracted which was higher at day 4 (Figure 5.12(A)). The absorbance ratio, 260nm/280nm ratio, showed a significant difference ($P<0.05$) in the RNA of the cells extracted at day 4 compared to day 6 (Figure 5.12(B)). The ratio reading was acceptable as good quality as ratio readings were in range (~2.0). The intact 18S and 28S rRNA bands were visible on the non-denaturing agarose gel electrophoresis, which was considered for good quality indication for RNA (Figure 5.13).

A)



B)

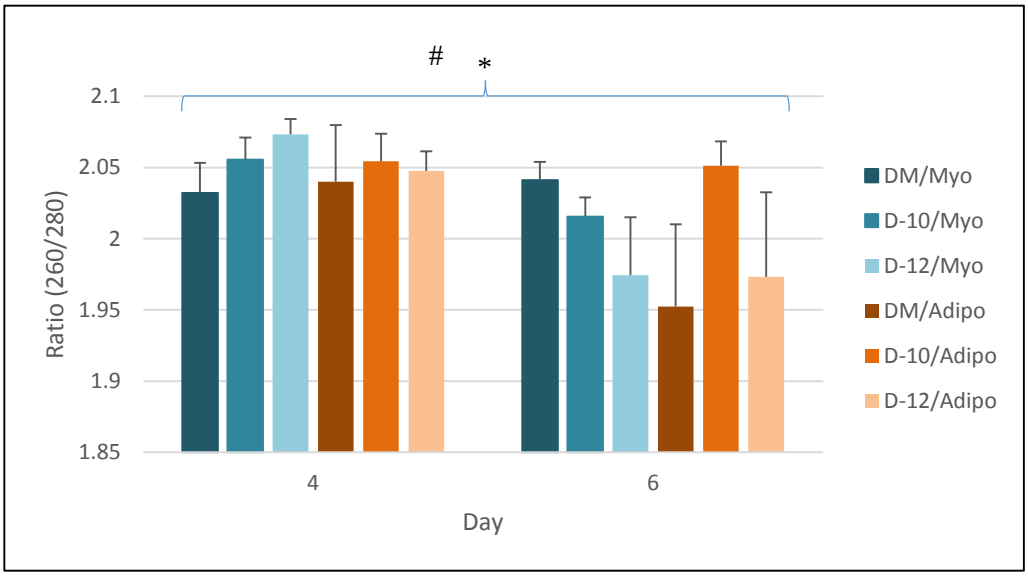
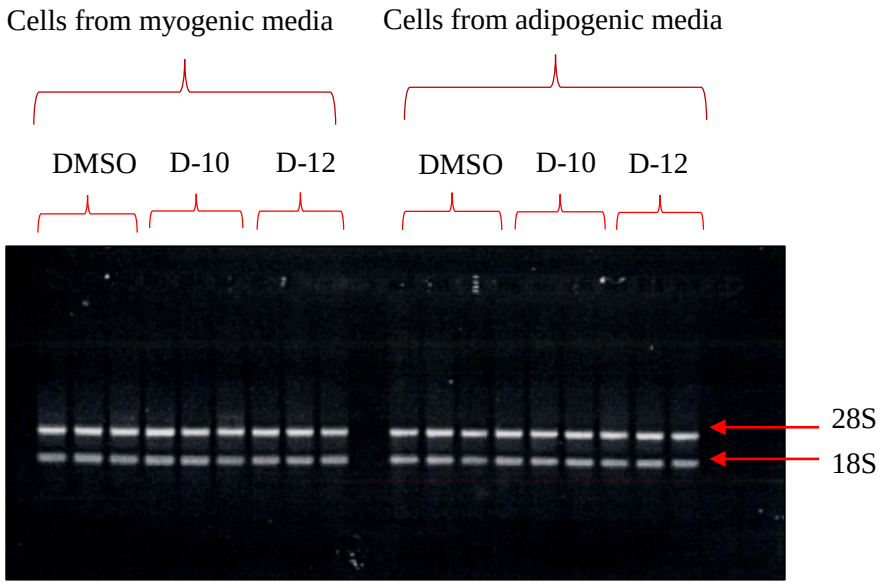


Figure 5.12: Dose-dependent effects of treatment either DMSO or $1,25(OH)_2D_3$ on (A) total RNA concentration and (B) absorbance ratio 260/280 in C2C12 cells exposed to adipogenic (Adipo) and myogenic (Myo) media. Cells were incubated in media containing either DMSO (DM), 10^{-10} M $1,25(OH)_2D_3$ (D-10) or 10^{-12} M $1,25(OH)_2D_3$ (D-12) from the point of 70-80% confluence (marked as day 0). The RNA was extracted from the cells at day 4 and day 6 of differentiation, with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of days of treatment (Day 4 vs Day 6).
‡ indicates effect of media (Adipo vs Myo).
*means significant different at $P<0.05$.
NS means no significant different.

A)



B)

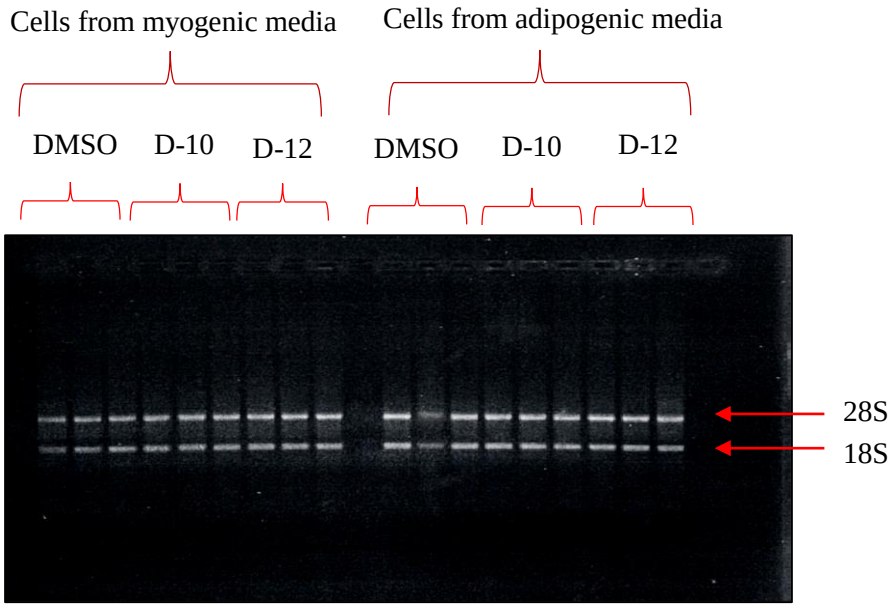


Figure 5.13: Dose-dependent effects of treatment either DMSO or 1,25$(OH)_2D_3$ on RNA integrity at (A) day 4 and (B) day 6 of differentiation. The RNA integrity in C2C12 cells was assessed using non-denaturing agarose gel electrophoresis. Cells were incubated in media containing either DMSO, 10^{-10} M 1,25$(OH)_2D_3$ (D-10) or 10^{-12} M 1,25$(OH)_2D_3$ (D-12) from the point of 70-80% confluence (day 0). The cells were scraped and extracted for RNA at day 4 and day 6 of differentiation. n=3 per treatment group. Two distinct bands represent 18S and 28S rRNA.

5.2.3.3.2 Dose-dependent effects of treatment either DMSO and 1,25(OH)$_2$D$_3$ (day 4 and day 6) in either myogenic and adipogenic media on lipid droplets accumulation

The accumulation of lipid droplet formation was confirmed in the cells incubated with adipogenic media, and were non-detectable in myogenic media (Figure 5.15). There was no significant interaction between the day of treatment and the treatment with 1,25(OH)$_2$D$_3$ on the accumulation of lipid droplets. However, there was a significant increase ($P<0.05$) of lipid droplets formation on day 6 compared with day 4, irrespective of the treatment effect (Figure 5.14).

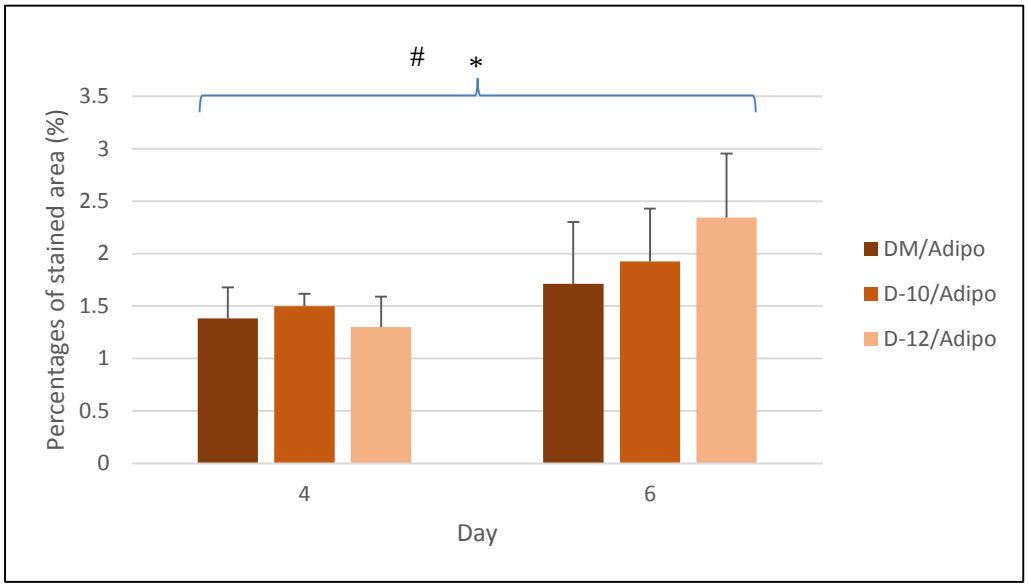


Figure 5.14: Dose-dependent effects of treatment either DMSO or 1,25(OH)$_2$D$_3$ on lipid droplets accumulation in C2C12 cells. Cells were exposed to adipogenic (Adipo) media containing either DMSO (DM), 10^{-10} M 1,25(OH)$_2$D$_3$ (D-10) or 10^{-12} M 1,25(OH)$_2$D$_3$ (D-12) from the point of 70-80% confluence (day 0) for 6 days, with n=3 per treatment group. After treatment, the cells were stained with Oil Red-O at day 4 (A) and day 6 (B) and the lipid droplets formation was quantified using Image J software. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of days of treatment (Day 4 vs Day 6).
*means significant different at $P<0.05$.

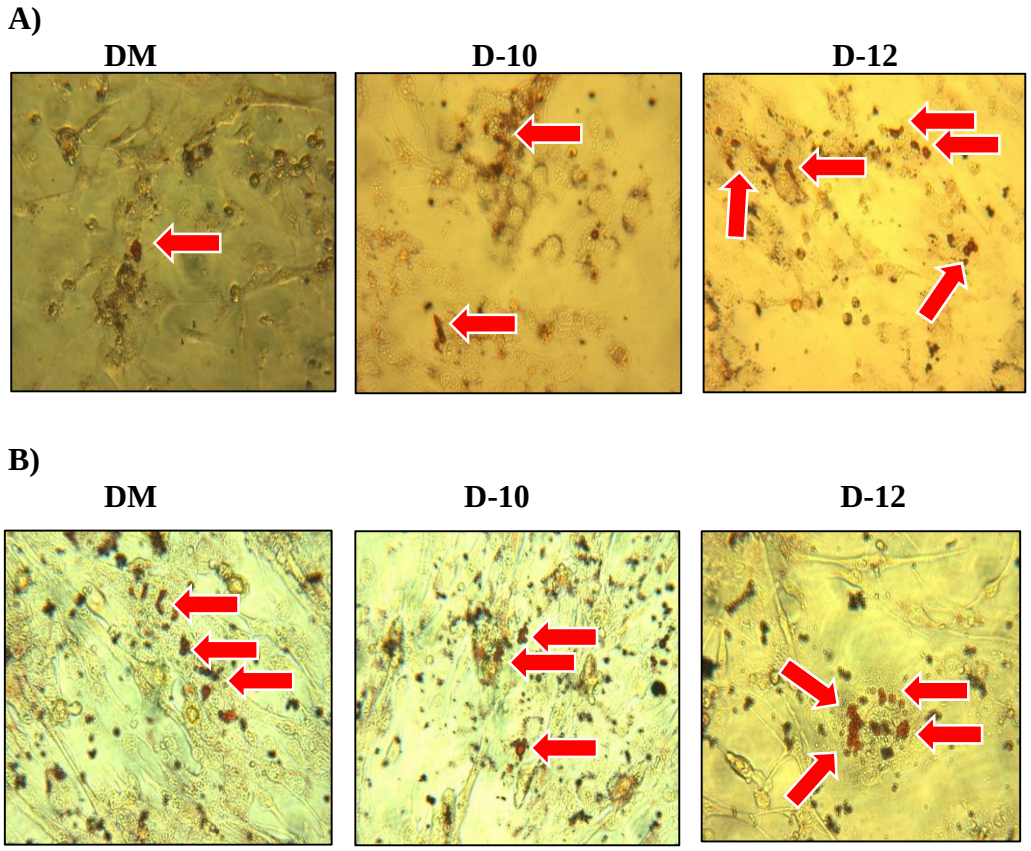


Figure 5.15: Dose-dependent effects of treatment either DMSO or $1,25(OH)_2D_3$ on lipid droplets accumulation in C2C12 cells. Cells were exposed to adipogenic media containing either DMSO (DM), 10^{-10} M $1,25(OH)_2D_3$ (D-10) or 10^{-12} M $1,25(OH)_2D_3$ (D-12) from the point of 70-80% confluence (day 0) for 6 days, with n=3 per treatment group. After treatment, the cells were stained with Oil Red-O at day 4 (A) and day 6 (B) and the images were visualized using the light microscope. The accumulation of the lipid droplets are indicated by the red arrows.

5.2.3.3.3 Dose-dependent effects of treatment either DMSO and 1,25(OH)$_2$D$_3$ (day 4 and day 6) in either myogenic and adipogenic media on mRNA expression related to myogenesis

There was no significant interaction between the effects of different dose of 1,25(OH)$_2$D$_3$ dose, time and media, neither was the effect of each individual factor on the oligreen concentration calibrated using myogenic pool. Therefore, oligreen measurements were used to normalise the RT-qPCR expression data (Figure 5.16).

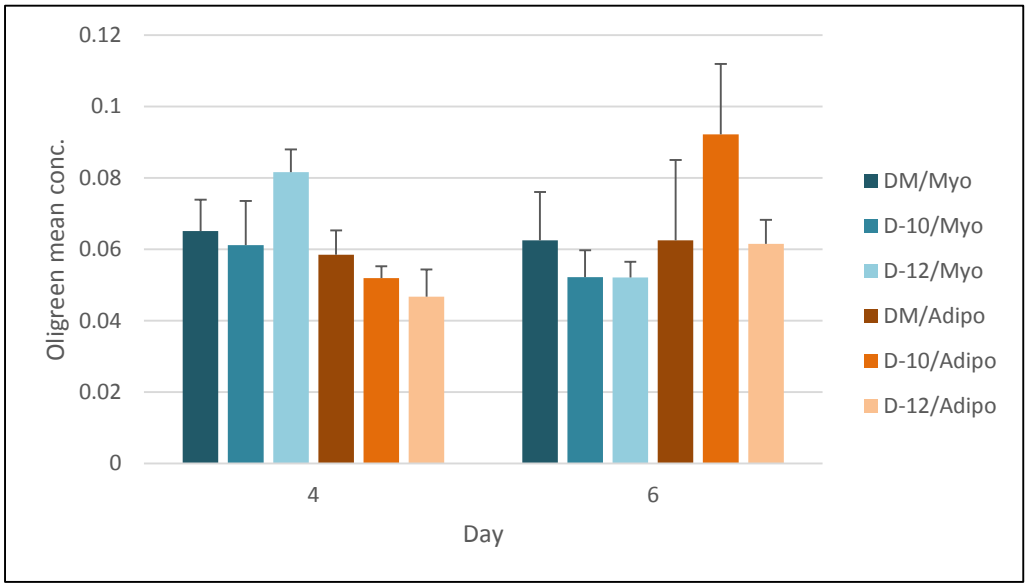


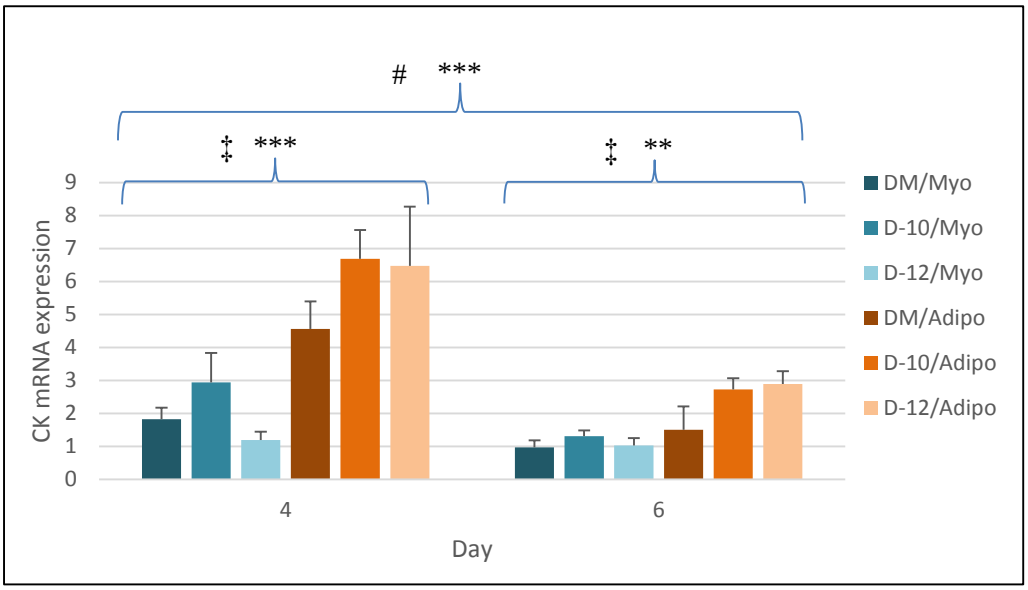
Figure 5.16: Dose-dependent effects of treatment either DMSO or 1,25(OH)$_2$D$_3$ on the oligreen concentration (corresponding to cDNA quantity) in C2C12 cells exposed to adipogenic (Adipo) and myogenic (Myo) media calibrated using myogenic pool. Cells were incubated in media containing either DMSO (DM (control), 10^{-10} M 1,25(OH)$_2$D$_3$ (D-10) or 10^{-12} M 1,25(OH)$_2$D$_3$ (D-12) from the point of 70-80% confluence (marked as day 0) for either 4 or 6 days with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM. No significant interaction and different between the effects of different dose of 1,25(OH)$_2$D$_3$ dose (DM vs D-10 vs D-12), days of treatment (Day 4 vs Day 6) and media (Adipo vs Myo).

For creatine kinase (CK) mRNA expression, there was a highly significant interaction between days of treatment and the incubation media ($P<0.01$). CK mRNA expression in cells treated with adipogenic media at day 4 was the highest irrespective of 1,25(OH)$_2$D$_3$ dose. In addition, when examining the single factor, there was a very highly significant increase of expression ($P<0.001$) in the cells

incubated in adipogenic media compared with in myogenic media as well as the very highly significant reduced of expression ($P<0.001$) on day 6 compared with day 4, without any effects of different doses of $1,25(OH)_2D_3$ (Figure 5.17(A)).

Similar effects were also observed in myogenin mRNA expression. There was a highly significant interaction between number days of treatment and the incubation media ($P<0.01$), but no other significant interactions, and a very highly significant increase of expression ($P<0.001$) in the cells incubated in adipogenic media compared in myogenic media as well as a very highly significant reduced of expression ($P<0.001$) on day 6 compared with day 4, without any effects of different dose of $1,25(OH)_2D_3$ (Figure 5.17(B)).

A)



B)

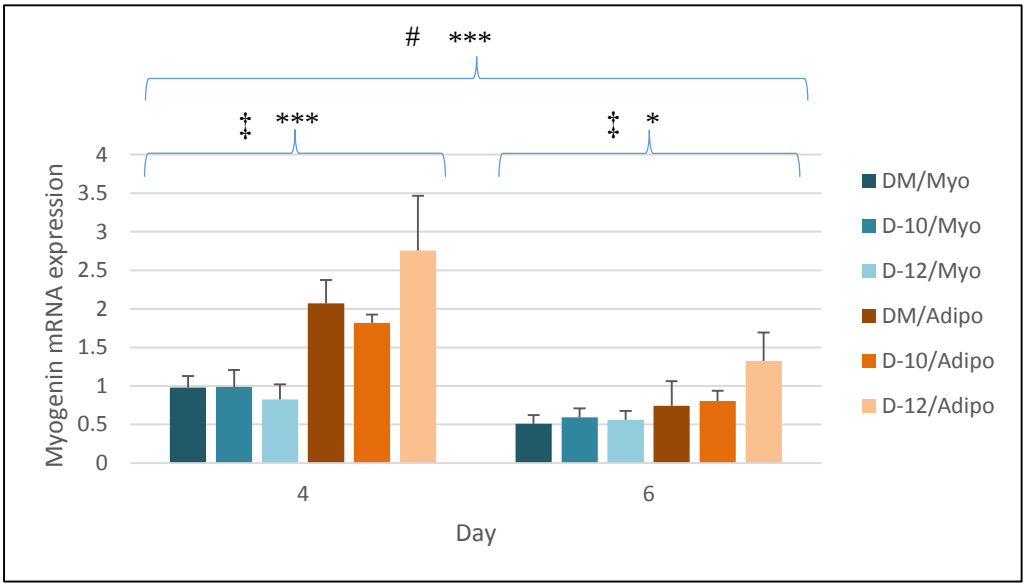


Figure 5.17: Dose-dependent effects of treatment either DMSO or $1,25(OH)_2D_3$ on the (A) CK mRNA expression and (B) myogenin mRNA expression in C2C12 cells exposed to adipogenic (Adipo) and myogenic (Myo) media. Cells were incubated in media containing either DMSO (DM), 10^{-10} M $1,25(OH)_2D_3$ (D-10) or 10^{-12} M $1,25(OH)_2D_3$ (D-12) from the point of 70-80% confluence (marked as day 0) for either 4 or 6 days with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of days of treatment (Day 4 vs Day 6).
‡ indicates to effect of media (Adipo vs Myo).
*means significant different at P<0.05.
**means highly significant different at P<0.01.
***means very highly significant different at P<0.001.

5.2.3.3.4 Dose-dependent effects of treatment either DMSO and 1,25(OH)$_2$D$_3$ (day 4 and day 6) in either myogenic and adipogenic media on mRNA expression related to adipogenesis

There were no significant effects of day of treatment (day 4 or day 6), treatment (DMSO and different dose of 1,25(OH)$_2$D$_3$) and incubation media (myogenic or adipogenic) on the oligreen concentration calibrated using the adipogenic pool. There was also no interaction between day of treatment, treatment and the incubation media. Therefore, oligreen measurements were used to normalise the RT-qPCR expression data (Figure 5.18).

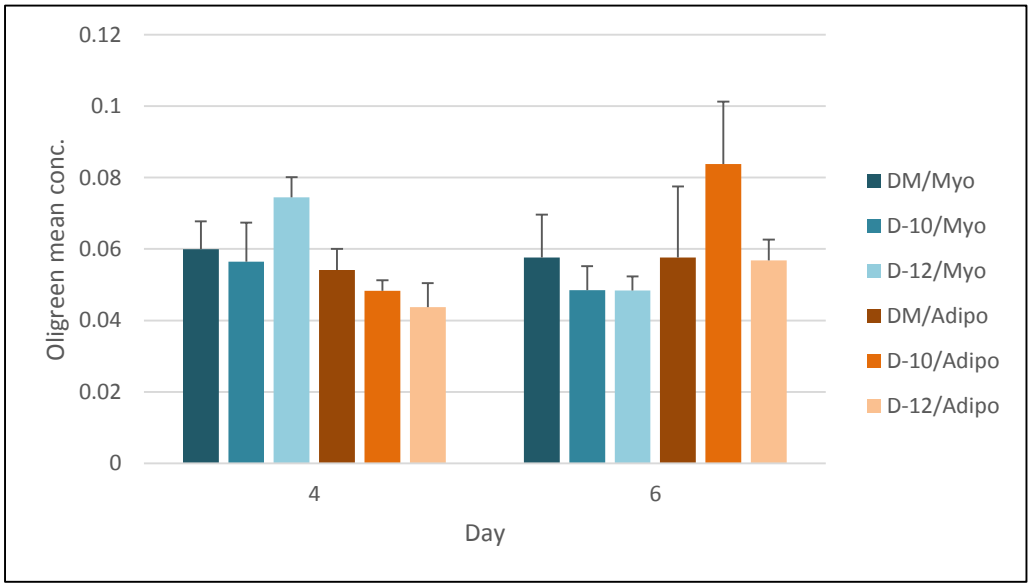


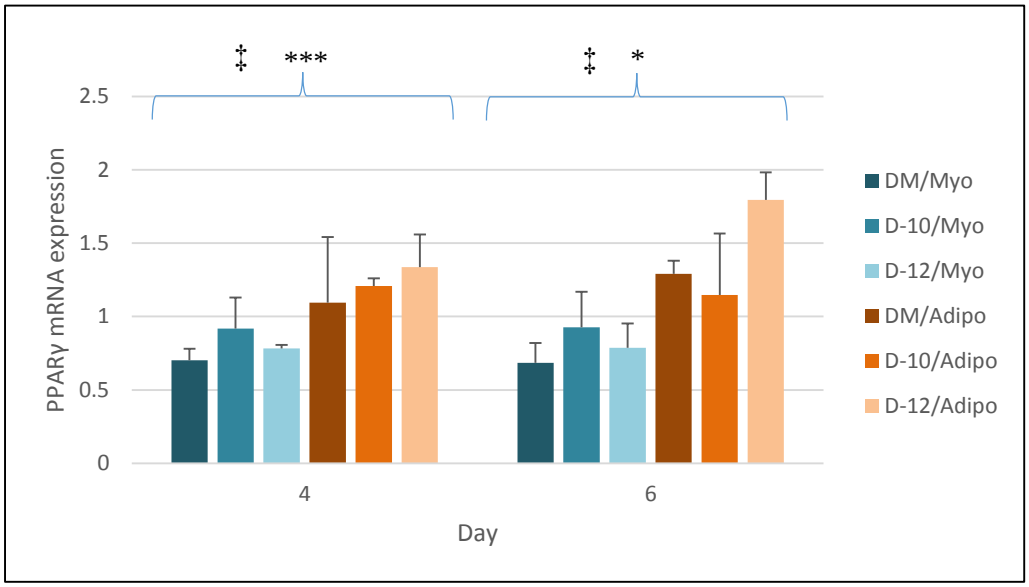
Figure 5.18: Dose-dependent effects of treatment either DMSO or 1,25(OH)$_2$D$_3$ on the oligreen concentration (corresponding to cDNA quantity) in C2C12 cells exposed to adipogenic (Adipo) and myogenic (Myo) media calibrated using adipogenic pool. Cells were incubated in media containing either DMSO (DM), 10^{-10} M 1,25(OH)$_2$D$_3$ (D-10) or 10^{-12} M 1,25(OH)$_2$D$_3$ (D-12) from the point of 70-80% confluence (marked as day 0) for either 4 or 6 days with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
No significant interaction and different between the effects of different dose of 1,25(OH)$_2$D$_3$ dose (DM vs D-10 vs D-12), days of treatment (Day 4 vs Day 6) and media (Adipo vs Myo).

For PPARγ gene expression, there was no interaction between the factors. However, there was a significant increased of expression ($P<0.01$) in the cells incubated in adipogenic media compared with myogenic media. There was no

effect of days of treatment or treatment with $1,25(OH)_2D_3$, irrespective of the other factors (Figure 5.19(A)).

There was no detectable expression of FABP4 in cells incubated in myogenic media irrespective of exposure to $1,25(OH)_2D_3$. However, when the C2C12 myoblasts were exposed to adipogenic media FABP transcripts were detectable. However, there was no significant effect of days of treatment, and treatments with DMSO or $1,25(OH)_2D_3$ (Figure 5.19(B)). The graph only showed the expression of the FABP4 in the adipogenic media as there was no expression of FABP4 in the myogenic media (data not shown).

A)



B)

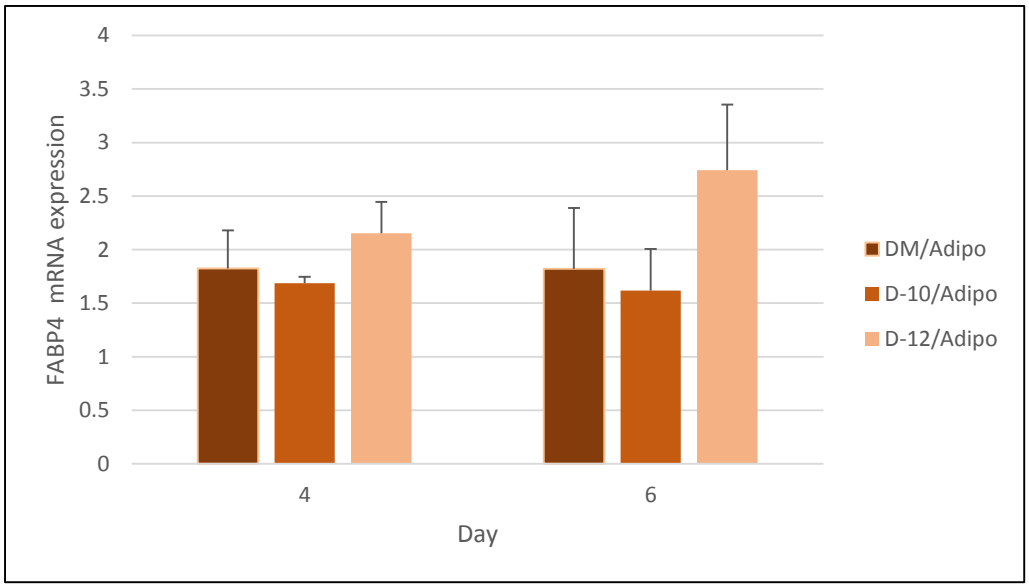


Figure 5.19: Dose-dependent effects of treatment either DMSO or 1,25$(OH)_2D_3$ on the (A) PPARγ mRNA expression and (B) FABP4 mRNA expression in C2C12 cells exposed to adipogenic (Adipo) and myogenic (Myo) media. Cells were incubated in media containing either DMSO (DM (control), 10^{-10} M 1,25$(OH)_2D_3$ (D-10) or 10^{-12} M 1,25$(OH)_2D_3$ (D-12) from the point of 70-80% confluence (marked as day 0) for either 4 or 6 days with n=3 per treatment group. The ANOVA were used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
‡ indicates effect of media (Adipo vs Myo).
*means significant different at P<0.05.
***means very highly significant different at P<0.001.
No significant interaction and different between the effects of different dose of 1,25$(OH)_2D_3$ dose (DM vs D-10 vs D-12) and days of treatment (Day 4 vs Day 6) in (B).

5.2.3.4 Summary

It appeared that the expressions of myogenic related genes were higher at day 4 and reduced towards the end of the differentiation periods. This probably reflected the development of the myotubes, day 4 being the time point where development was the most rapid. Interestingly, the mRNA expression of the myogenic related genes was increased in the adipogenic media incubation compared with myogenic media, along with induction of the adipogenic related mRNA expression, while retaining their myogenic properties. This might be a useful and important finding as this is analogous to *in vivo*, where there are adipocytes located within muscle tissue and the deposition of fat which is responsible for intramuscular fat. The mRNA expression was not affected by the dosage of the $1,25(OH)_2D_3$. Thus, in the next section, only one dosage of $1,25(OH)_2D_3$ was used along with the supplementation of the dibutyryl cyclic adenosine monophosphate (dbcAMP) and Des (1-3) IGF-1 in the adipogenic culture.

5.2.4 Effects of day of treatment with dibutyryl cyclic adenosine monophosphate (dbcAMP) and Des (1-3) IGF-1, in adipogenic media on myogenesis- and adipogenesis- related mRNA expressions

5.2.4.1 Introduction

As indicated in the previous sections, the incubation of differentiating C2C12 cells with adipogenic media showed the characteristics of differentiating to muscle cells as they expressed genes associated with myogenesis. In previous work, it had been demonstrated that there was an increased activity of creatine kinase (Portier et al., 1999), a mark of muscle differentiation. However, as demonstrated in the previous section, the addition of adipogenic media resulted in the increase, or induction, of the expression of genes associated with fatty acid metabolism in adipocytes. This was associated with the deposition of fat droplets. Previous work from our laboratories had also demonstrated that the addition of active vitamin D to the media had the effect of accentuating both the deposition of fat droplets and the expression of genes associated with fatty acid metabolism (Ryan et al., 2013). Although in the previous section, addition of either both doses of $1,25(OH)_2D_3$ did not statistically affect the PPARγ and FABP4 mRNA expressions, it did appear that the cells exposed to the adipogenic media did increase their fatty acid metabolism which resulted in the development of cells exhibiting a phenotype, which was potentially representative of *in vivo* muscle in adult animals. Therefore, the experiment described in the next section went on to utilise the cells expressing "adipo-like" characteristics in culture to determine whether the agents demonstrated as stimulating a decreasing fat deposition in muscle *in vivo* would exhibit the same effects in these "adipo-like" muscle cells in culture. As in the previous section, the dosage of the $1,25(OH)_2D_3$ did not affect any expression of the target mRNAs. Therefore, the dosage of 10^{-12} M was used in this section, which is the intermediate of low physiological dosage (10^{-13} and 10^{-11} M), and previously had appeared to result in the increased accumulation of the lipid droplets and upregulation of the adipogenic genes in C2C12 cells incubated in adipogenic media (Ryan et al., 2013). This dosage was supplemented to the adipogenic differentiation media during a preincubation step to induce the expression of adipogenic genes and associated with lipid

droplet accumulation. Following this preincubation step, the media was replaced with adipogenic media without 1,25$(OH)_2D_3$ and the cells were treated with dibutyryl cyclic adenosine monophosphate (dbcAMP) or Des (1-3) IGF-1 supplementations. dbcAMP is an analogue for the cyclic AMP, which is generated following beta-agonist activation of beta-adrenergic receptors and subsequently stimulates protein kinase A (PKA) activity (Mills and Liu, 1990). Meanwhile, Des (1-3) IGF-1 is an analogue for the insulin like growth factor-1 (IGF-1), which is increased in the plasma following growth hormone stimulation (Ballard et al., 1996).

The hypothesis of this experiment was that C2C12 incubated in adipogenic media with or without 1,25$(OH)_2D_3$ would induced deposition of fat droplets and have the gene expressions associated with the formation of adipocytes whilst maintaining their myogenic characteristics. Following this preincubation, this mixed cell population would then respond to the analogues of metabolic modifiers (dbcAMP or Des (1-3) IGF-1) in manner similar to the changes in gene expressions found in the muscles of animals treated with BA and GH.

5.2.4.2 Materials and Methods

5.2.4.2.1 Preincubation with DMSO and 1,25$(OH)_2D_3$

To investigate the effect and interaction of time-dependent and treatment either with dbcAMP or Des (1-3) IGF-1 on gene expression during C2C12 cell differentiation, the cells were grown in normal growth media, passaged and induced with the adipogenic differentiation. The commencement of the preincubation during adipogenic differentiation was also supplemented with either 0.1% (v/v) dimethyl sulfoxide (DMSO) (DM), or 10^{-12} M of 1,25$(OH)_2D_3$ (Vit D+) (Sigma, UK), according to Figure 5.20. This was marked as day 0 of preincubation differentiation. The differentiating cells in the preincubation media were harvested and scraped for RNA on day 4 and day 6 for RT-qPCR analysis, as well as the Oil Red O staining for the lipid droplets quantification. The protocols for cell growth and differentiation, passaging, Oil Red-O staining and gene expressions analysis were already described in the Section 3.3.

5.2.4.2.2 Treatment with DMSO, dbcAMP, and Des (1-3) IGF-1

After day 6 preincubation with DM or Vit D+, all the differentiating C2C12 cells were switched to adipogenic media containing the recombinant human insulin like growth factor-1, Des (1-3) IGF-1 (Autogen Bioclea, Bioscience UK Ltd) or dibutyryl cyclic adenosine monophosphate (dbcAMP) (Sigma-Aldrich, UK) or DMSO. The Des (1-3) IGF-1 was dissolved in 0.1 M acetic acid for 100 µg/ml concentration for the stock solution. Upon addition into the differentiation media, the stock solution of Des (1-3) IGF-1 was diluted with PBS to a concentration 100 ng/ml, followed by addition into the differentiation media for a concentration of 20ng/ml, according to Figure 5.21. The dbcAMP was added into the differentiation media for a final concentration of 1 mM, according to Figure 5.21. This was marked as day 0 for the treatment. The cells were harvested and scraped at day 1, 2, and 3 after the addition of these treatments for the RNA extraction. The cells were stained with Oil Red O staining for the lipid droplets quantification at day 3 of differentiation.

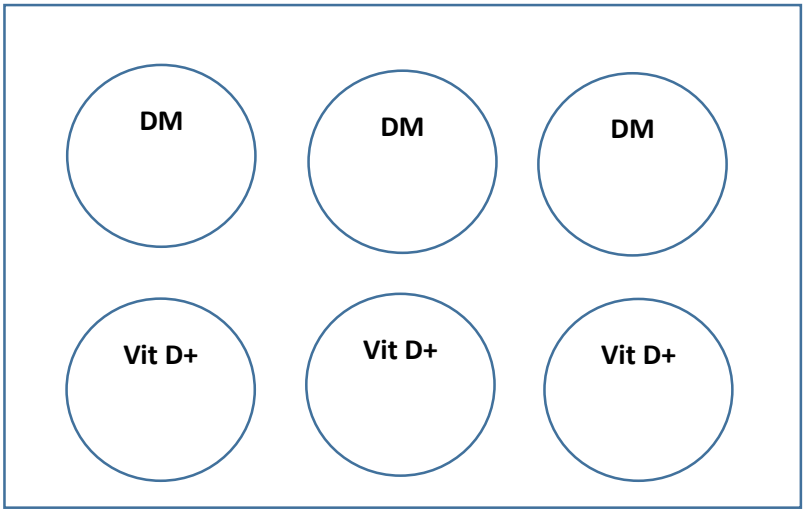


Figure 5.20: The preincubation layout with treatments on 6-well plate. The above layout showed the position of the treatment with DMSO (DM), or 10^{-12} M of $1,25(OH)_2D_3$ (vit D+) on the 6-well plate. This layout was designed to eliminate the effect of the plate (all factors on one plate), with n=3.

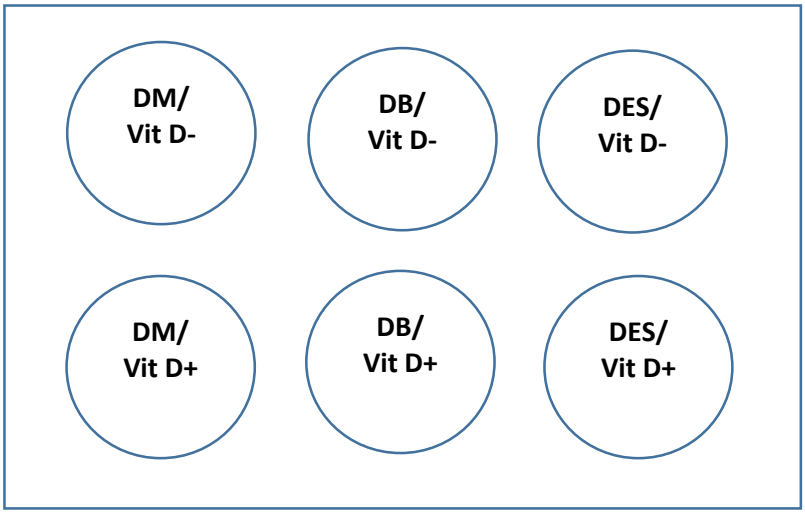


Figure 5.21: The incubation layout with treatments on 6-well plate. The above layout showed the position of the treatment with DMSO (DM), dbcAMP (DB), and Des (1-3) IGF-1 (DES), with indication that the cells were preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) on the 6-well plate. This layout was designed to eliminate the effect of the plate (all factors on one plate), with n=3.

5.2.4.3 Results

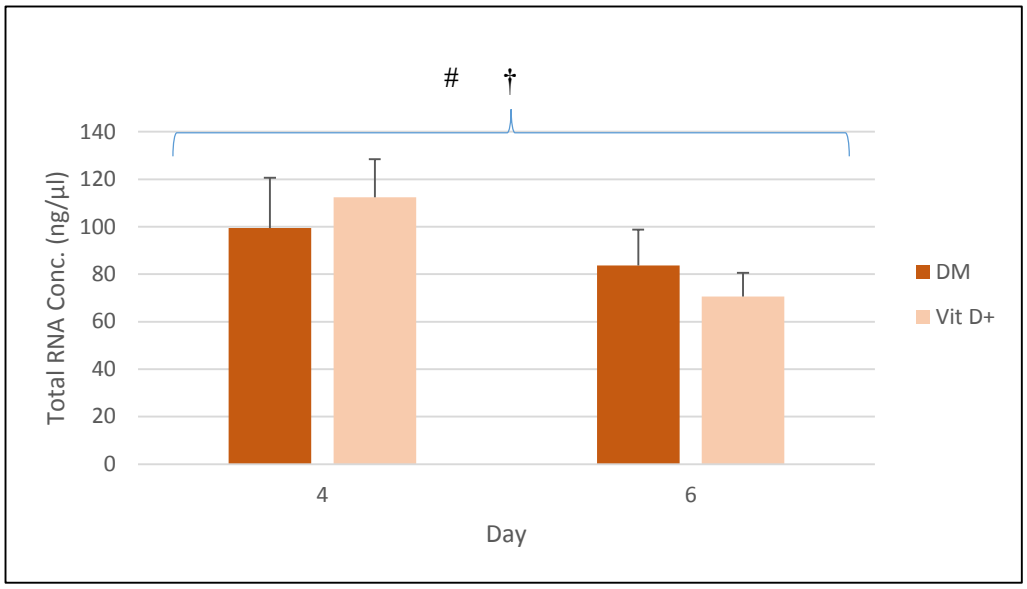
5.2.4.3.1 Effects of days of treatment with dbcAMP, Des (1-3) IGF-1, and $1,25(OH)_2D_3$ preincubation in adipogenic media on RNA quantity and quality.

During the preincubation step to induce fat droplet formation, before treated with the dbcAMP and Des (1-3) IGF-1, the total RNA from the C2C12 cells was extracted at two time points, day 4 and day 6 of differentiation in the adipogenic media, supplemented with either DMSO or $1,25(OH)_2D_3$ only. There was a trend ($P = 0.095$) of decreasing the total RNA concentration on day 6 compared to day 4 of differentiation, irrespective the treatment with DMSO or $1,25(OH)_2D_3$ (Figure 5.22(A)). Meanwhile, there was no effect of different treatments on the absorbance ratio, 260nm/280nm ratio (Figure 5.22(B)).

After the subsequent treatment with dbcAMP and Des (1-3) IGF-1, there was an interaction between day of treatment and the treatment either with dbcAMP and Des (1-3) IGF-1. There was also a significant reduced ($P<0.05$) of total RNA concentration from the cells that had been preincubated with $1,25(OH)_2D_3$ compared with non-preincubated cells. In addition, the cells treated with Des (1-

3) IGF-1 had a very highly significant lower ($P<0.001$) in total RNA concentration compared with the cells treated with DMSO and dbcAMP. Irrespective for treatments, the total RNA concentration was also significantly increased at day 2 after treatment, and reduced very highly significantly at day 3 ($P<0.001$) (Figure 5.24(A)). However, the absorbance ratio, 260nm/280nm ratio was not affected by either time of incubation, DMSO, dbcAMP, Des (1-3) IGF-1 or preincubation with $1,25(OH)_2D_3$ (Figure 5.24(B)).

A)



B)

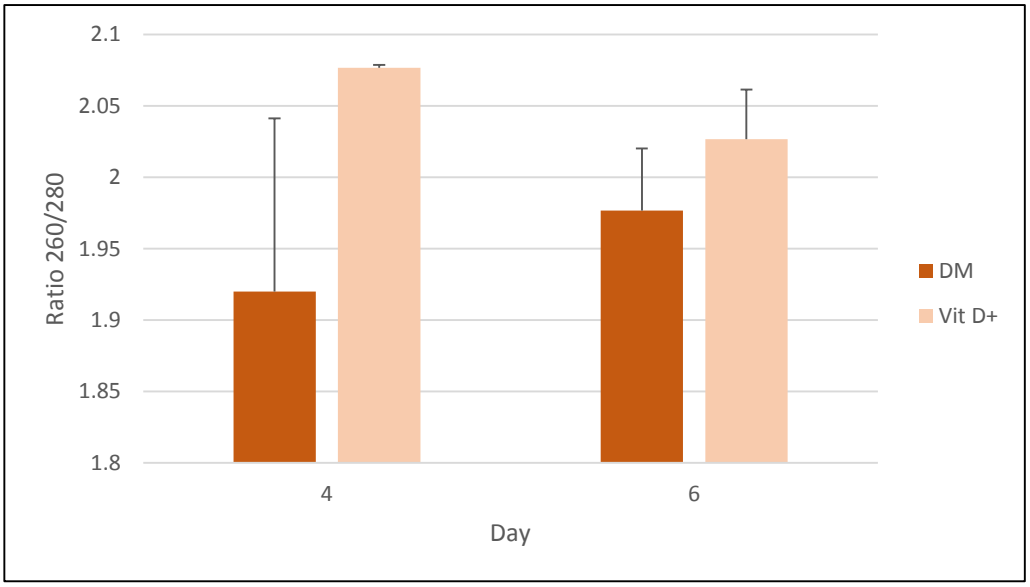


Figure 5.22: Effects of day of treatment, on (A) total RNA concentration and (B) absorbance ratio 260/280 in C2C12 cells. Cells were exposed in adipogenic differentiation media preincubated either DMSO (DM) or 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) from the point of 70-80% confluence (marked as day 0). The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test, with n=3 per treatment group. The error bars are ± SEM.
# indicates effect of time of incubation (Day 4 vs Day 6).
† means trend at $P<0.1$.
No significant interaction and different between the effects of treatments (DM vs Vit D+) and time of incubation (Day 4 vs Day 6) in (B).

The individual RNA quality from the cells before and after treatment were shown in Figure 5.23 and Figure 5.25, respectively. The intact 18s and 28s rRNA bands were clearly visible, by the non-denaturing agarose gel electrophoresis, which was considered for good quality indication for RNA.

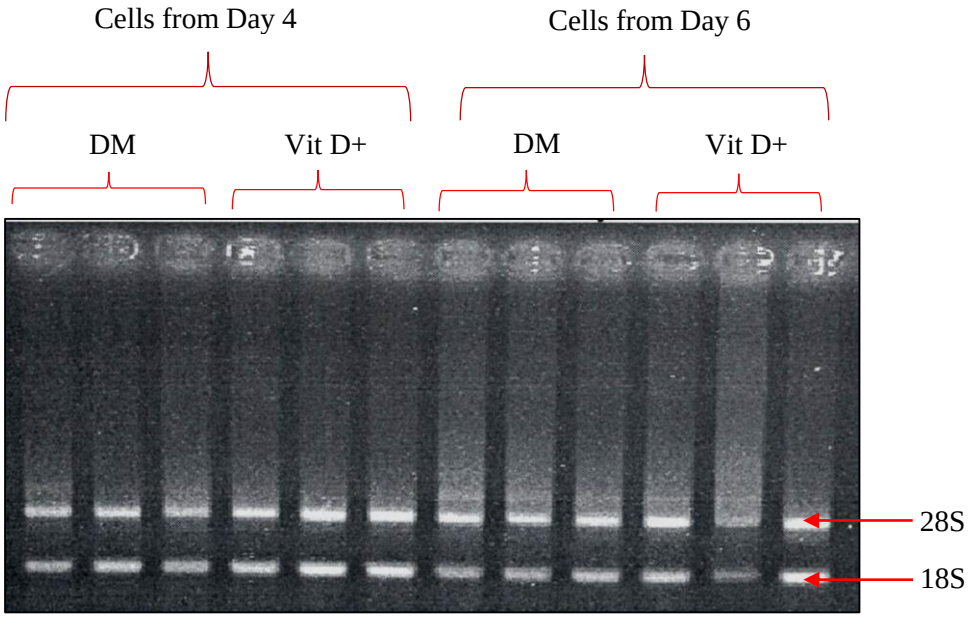
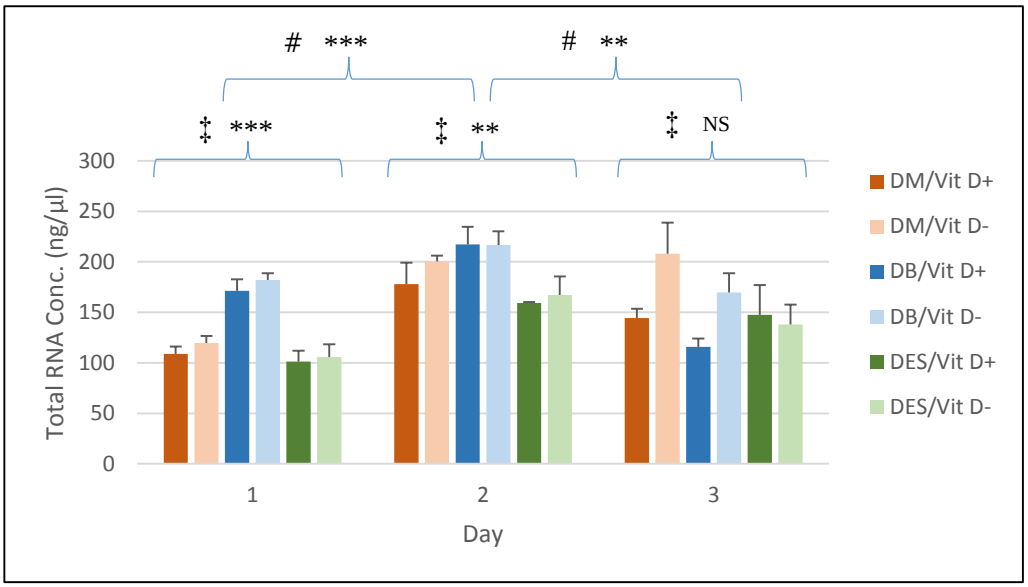


Figure 5.23: Effects of day of treatment on RNA integrity from the cells extracted at day 4 and day 6 of differentiation before treated with DMSO, dbcAMP and Des (1-3) IGF-1. The RNA integrity in C2C12 cells was assessed using non-denaturing agarose gel electrophoresis. Cells were exposed in adipogenic differentiation media preincubated either DMSO (DM), or 10^{-12} M $1,25(OH)_2D_3$ (Vit D+) from the point of 70-80% confluence (day 0). The cells were scraped and extracted for RNA at day 4 and day 6 of differentiation with n=3 per treatment group. Two distinct bands represent 18S and 28S rRNA.

A)



B)

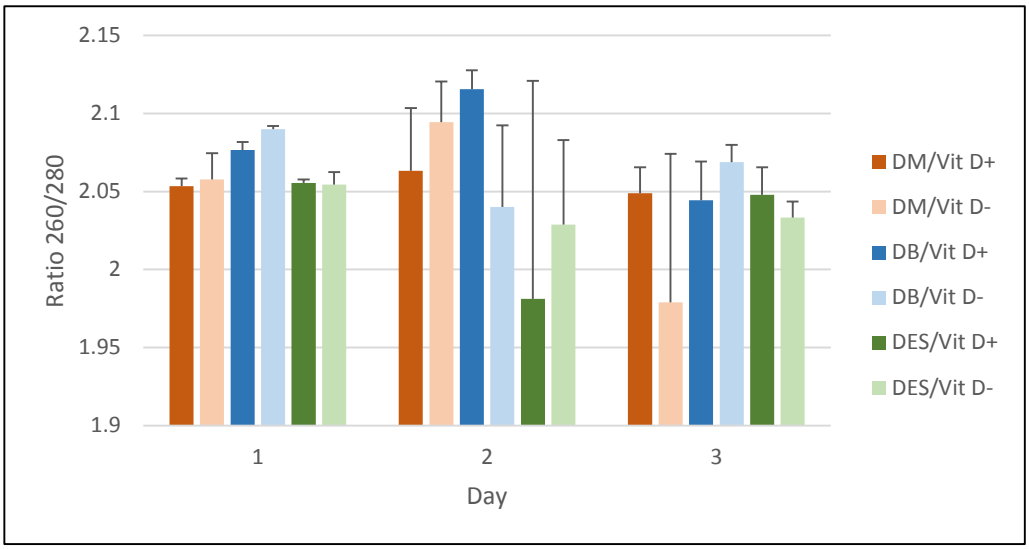


Figure 5.24: Effects of day of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on (A) total RNA concentration and (B) absorbance ratio 260/280 in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of time of incubation (Day 1 vs Day 2 vs Day 3).
‡ indicates effect of treatments (DM vs DB vs DES).
**means highly significant different at P<0.01.
***means very highly significant different at P<0.001.
NS means no significant different.
No significant interaction and different between the effects of treatments (DM vs DB vs DES) and time of incubation (Day 1vs Day 2 vs Day 3) in (B).

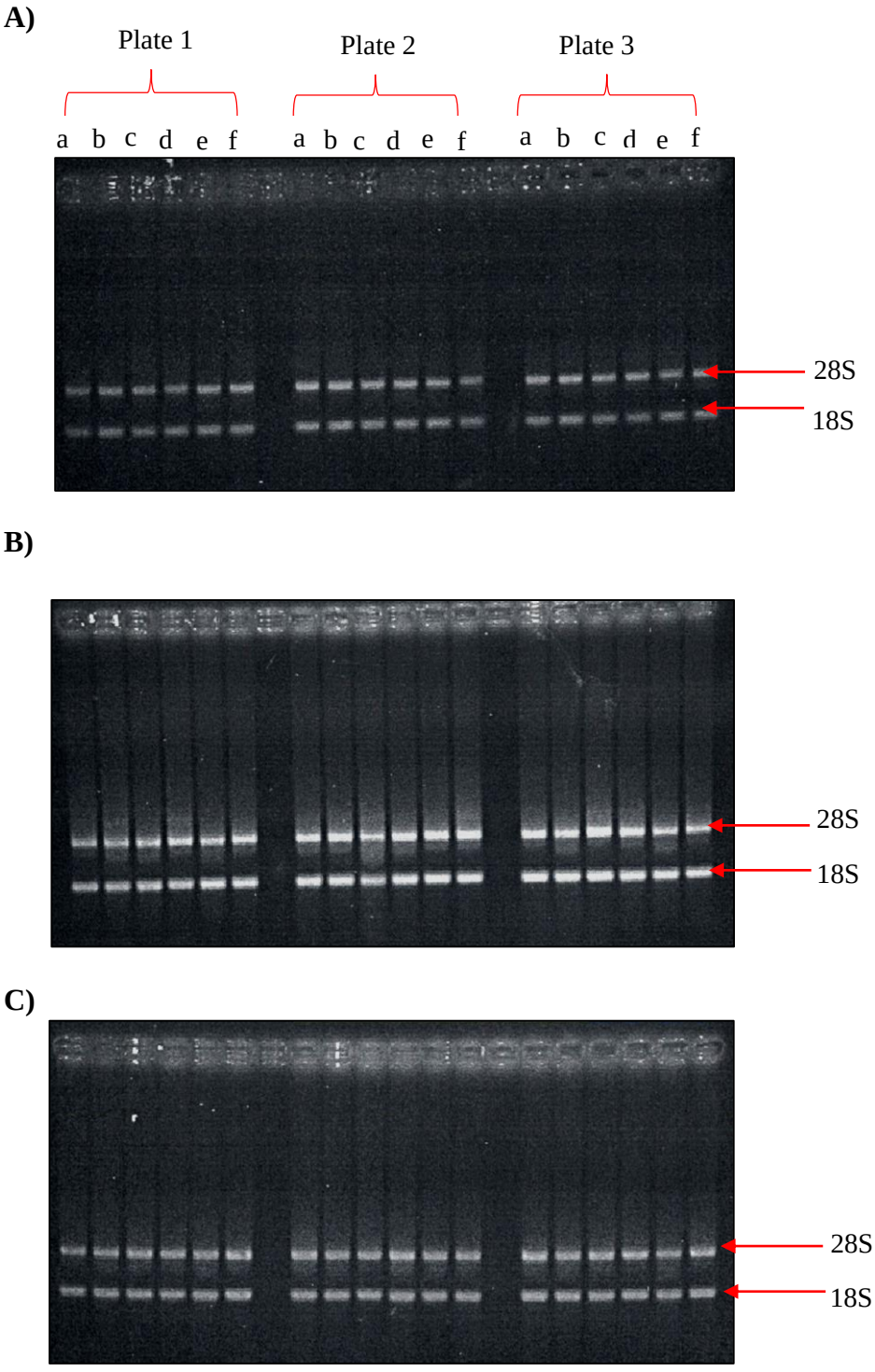


Figure 5.25: Effects of day of treatment on RNA integrity from the cells extracted at (A) day 1, (B) day 2 and (C) day 3 of treatment. The RNA integrity in C2C12 cells was assessed using non-denaturing agarose gel electrophoresis. Cells were exposed in adipogenic differentiation media containing either (a) DMSO only, (b) dbcAMP only, (c) Des (1-3) IGF-1 only, (d) DMSO after preincubated with 10^{-12} M $1,25(OH)_2D_3$, (e) dbcAMP after preincubated with 10^{-12} M $1,25(OH)_2D_3$ or (f) Des (1-3) IGF-1 after preincubated with 10^{-12} M $1,25(OH)_2D_3$, from the point of 70-80% confluence (day 0). The cells were scraped and extracted for RNA at day 1, 2 and 3 of treatment. n=3 per treatment group. Two distinct bands represent 18S and 28S rRNA.

5.2.4.3.2 Preincubation: The lipid droplets formation and mRNA expression before treatment with dbcAMP and Des (1-3) IGF-1

The first component of the experiment was to generate the C2C12 cells in culture which were induced to express "adipo-like" characteristics. As described in the previous section C2C12 cells were exposed to adipogenic media in the presence of 10^{-12} M $1,25(OH)_2D_3$ or the control (DMSO) for up to 6 days, this was considered to be the preincubation step. A subsection of cells was taken to establish the changes in gene expression of both genes associated with myogenesis and adipogenesis. The list of genes assessed are indicated in Table 5.1.

Table 5.1: Genes assessed for transcript expression in C2C12 cells exposed to adipogenic media in the presence of 10^{-12} M of $1,25(OH)_2D_3$

Type	Gene symbol	Name of product
Lipogenic	ACC	Acetyl-CoA Carboxylase Alpha (ACACA)
	FAS	Fatty Acid Synthase (FASN)
Lipolytic	ATGL	Patatin Like Phospholipase Domain Containing 2 (PNPLA2)
	HSL	Lipase E, Hormone Sensitive Type
Adipocyte Transcription Factors	PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
	CD36	Cluster Determinant 36
	C/EBPα	CCAAT/Enhancer Binding Protein Alpha
	FABP4	Fatty Acid Binding Protein-4
Muscle Differentiation Markers	CKM	Creatine Kinase, M-type
Myogenic Regulatory Factors (MRFs)	MYF5	Myogenic Factor 5
	MYOD	Myogenic Differentiation 1 (MYOD1)

	MYOG	Myogenin
Myofibrillar	MYH7	Myosin Heavy Chain I
Markers	MYH2	Myosin Heavy Chain IIa
	MYH4	Myosin Heavy Chain IIb
	MYH1	Myosin Heavy Chain IIx

After preincubation, the presence of lipid droplets was confirmed by the Oil Red-O staining (Figure 5.27). There was no significant interaction between the time of incubation and treatment with 10^{-12} M $1,25(OH)_2D_3$ on lipid droplets accumulation. There was only a significant increase ($P<0.05$) of lipid droplet formation on day 6 compared with day 4 without any effects of the DMSO (DM) or $1,25(OH)_2D_3$ (Vit D+) (Figure 5.26).

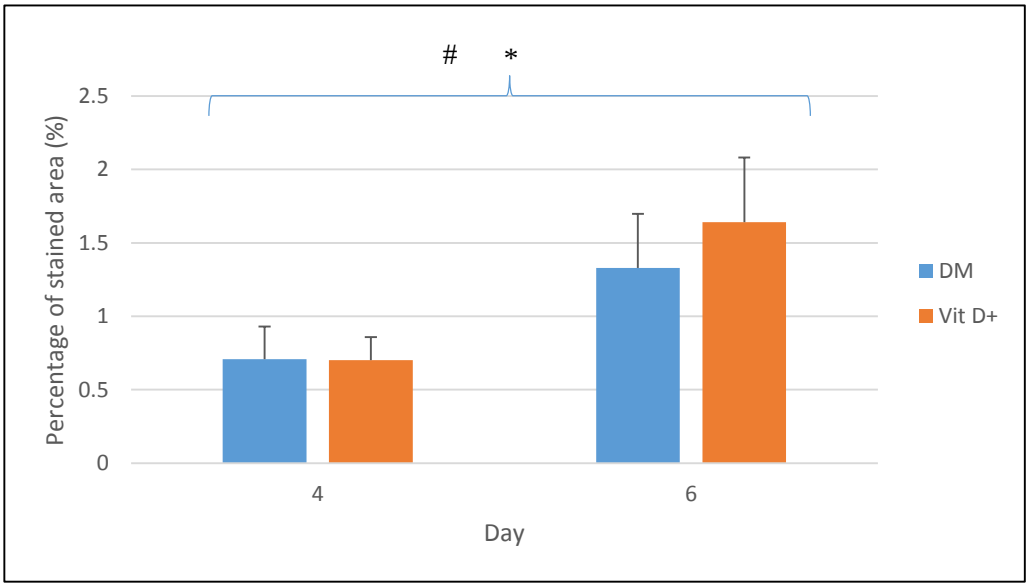


Figure 5.26: Effects of treatment with either DMSO (DM) or $1,25(OH)_2D_3$ (Vit D+) on the percentage area stained with Oil Red-O for lipid droplets in C2C12 cells. Cells were exposed in adipogenic differentiation media preincubated either DMSO (DM) or 10^{-12} M $1,25(OH)_2D_3$ (D3) from the point of 70-80% confluence (day 0) for 6 days. The Oil Red O staining was performed on day 4 and day 6, with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of time of incubation (Day 4 vs Day 6).
*means significant different at $P<0.05$.

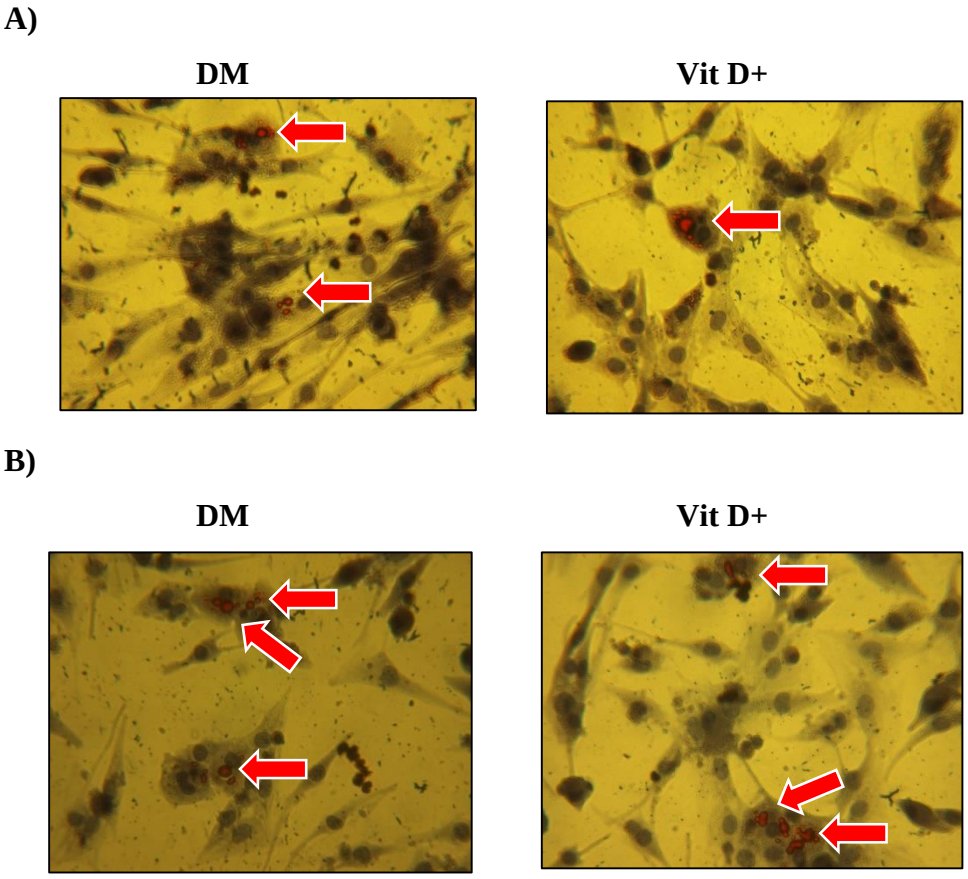


Figure 5.27: Effects of treatment either DMSO (DM) or 1,25$(OH)_2D_3$ (Vit D+) on lipid droplets accumulation in C2C12 cells. Cells were exposed in adipogenic differentiation media preincubated either DMSO or 10^{-12} M 1,25$(OH)_2D_3$ from the point of 70-80% confluence (day 0) for 6 days with n=3 per treatment group. After treatment, the cells were stained with Oil Red-O at day 4 (A) and day 6 (B) and the images were visualized using the light microscope. The accumulation of the lipid droplets were indicated by the red arrows.

The similar pattern was demonstrated on the FABP4 expression. There was no interaction between the number of days of incubation and treatment with DMSO or 1,25$(OH)_2D_3$ on FABP4 expression. However, there was a highly significant increased ($P<0.01$) in expression of FABP4 on day 6 compared with day 4 without any effect of the treatment with DMSO or 1,25$(OH)_2D_3$ (Figure 5.28).

There were no effects between the day and the treatment with DMSO or 1,25$(OH)_2D_3$ on the expression of any other genes measured (Table 5.1) (data not shown). Thus, it suggested that the treatment with 1,25$(OH)_2D_3$ did not appear to accentuate the effects of the adipogenic media unlike in the previous studies

(Ryan et al., 2013). However, it was apparent that adipogenic media did increase the deposition of fat droplets in the cells.

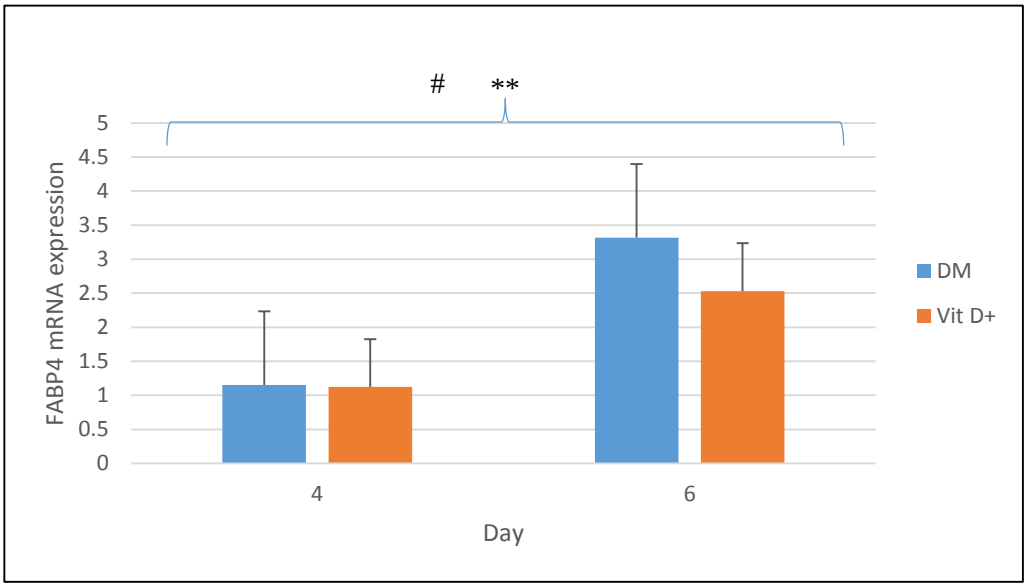


Figure 5.28: Effects of treatment with either DMSO (DM) or 1,25$(OH)_2D_3$ (Vit D+) on the mRNA expression of FABP4 in C2C12 exposed to adipogenic media. Cells were exposed in adipogenic differentiation media preincubated either DMSO (DM) or 10^{-12} M of 1,25$(OH)_2D_3$ (Vit D+) from the point of 70-80% confluence (day 0) for either 4 or 6 days, before treatment with dbcAMP and Des (1-3) IGF-1, with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of time of incubation (Day 4 vs Day 6).
**means highly significant different at P<0.01.

5.2.4.3.3 Post-incubation: The lipid droplets accumulation after treatment with dbcAMP and Des (1-3) IGF-1

The preincubation of C2C12 cells with adipogenic media for 6 days in the presence or absence of 10^{-12} M 1,25$(OH)_2D_3$ resulted in cells accumulating lipid but at the same time retaining their myogenic capacity. Following this pre-exposure period the cells were switched to the same adipogenic media (no 1,25$(OH)_2D_3$ added) and the additional agents were added. The two agents selected were those that would stimulate signalling pathways activated by beta-adrenergic agonists or growth hormone, dibutyryl cyclic adenosine monophosphate (dbcAMP) or Des (1-3) insulin growth factor -1 (Des (1-3) IGF-

1), respectively. The mature C2C12 cells were incubated with these agents for 3 days.

As indicated in Figure 5.30, lipid droplets were accumulated in the cells incubated in adipogenic media in the presence of DMSO for 3 days following the 6 day preincubation period. There was a very highly significant interaction between the treatment and the preincubation with $1,25(OH)_2D_3$ ($P<0.001$), with DMSO only having the highest area of lipid droplets. Meanwhile, in comparison to the cells incubated in DMSO in adipogenic media without preincubation with $1,25(OH)_2D_3$ supplementation, all the other treatments gave a very highly significant lower ($P<0.001$) of lipid droplets in the cells (Figure 5.29).

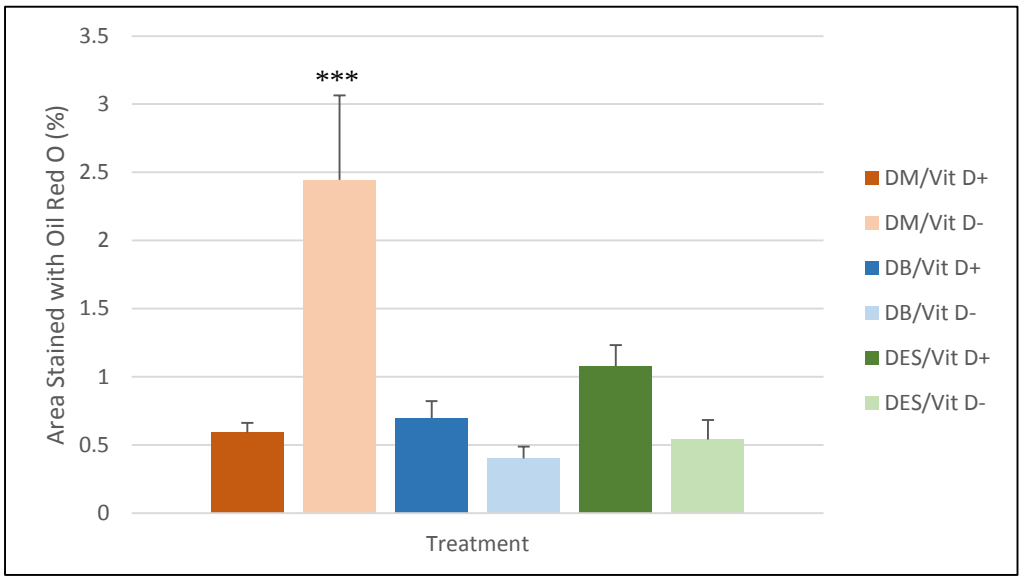


Figure 5.29: Effects of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on percentage area stained with Oil Red O for lipid droplets in C2C12 cells. The C2C12 cells were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. On day 3 of differentiation, the cells were stained with Oil Red-O. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
***means significant different at $P<0.001$, by Bonferroni's test, relative to other groups.

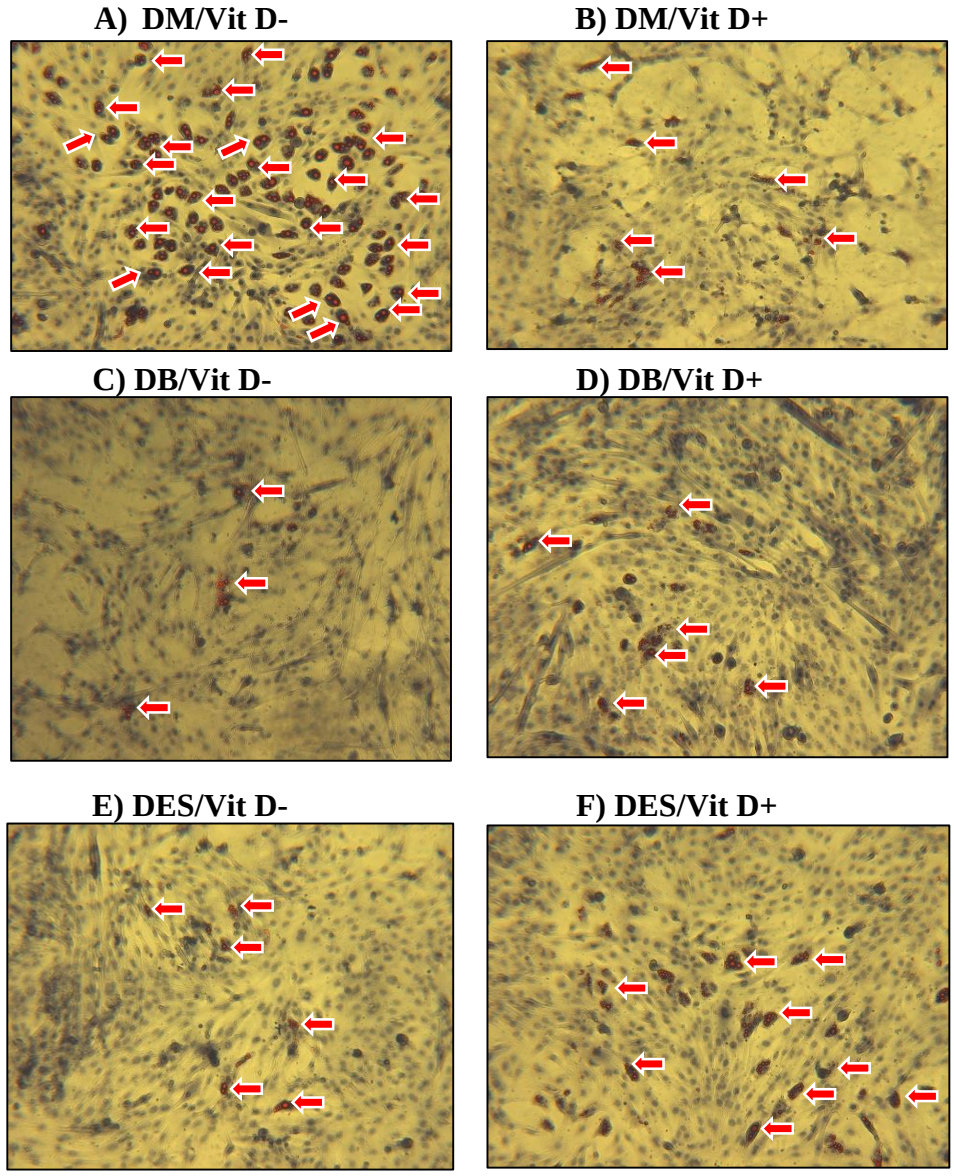


Figure 5.30: Effects of treatment either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on lipid droplets accumulation in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. On day 3 of differentiation, the cells were stained with Oil Red-O and the images were visualized using the light microscope. The lipid droplets accumulation were indicated by the red arrows.

5.2.4.3.4 mRNA expressions after treatment with dbcAMP and Des (1-3) IGF-1

Following a preincubation of C2C12 in adipogenic media with or without $1,25(OH)_2D_3$, the subsequent 3-day treatment of preincubated C2C12 cells was

done in adipogenic media with no 1,25$(OH)_2D_3$ present. During this 3-day period, cells were treated with DMSO, dbcAMP or Des (1-3) IGF-1, and the gene expression of mRNA transcripts associated with lipid metabolism, including lipogenesis, lipolysis, and adipocyte metabolism, were assessed on each day of treatment (Table 5.1). In addition, the expression of the mRNA related with muscle development, including myogenic regulatory factors, muscle differentiation and myofibrillar markers, were assessed as well (Table 5.1). Assessment was made whether there was an effect on time of incubation (time) or the preincubation with or without 1,25$(OH)_2D_3$ (media) or treatment with the agents which activate pathways simulated by beta-adrenergic agonists or growth hormone (treatments), dibutyryl cyclic adenosine monophosphate (dbcAMP) or Des (1-3) IGF-1 respectively.

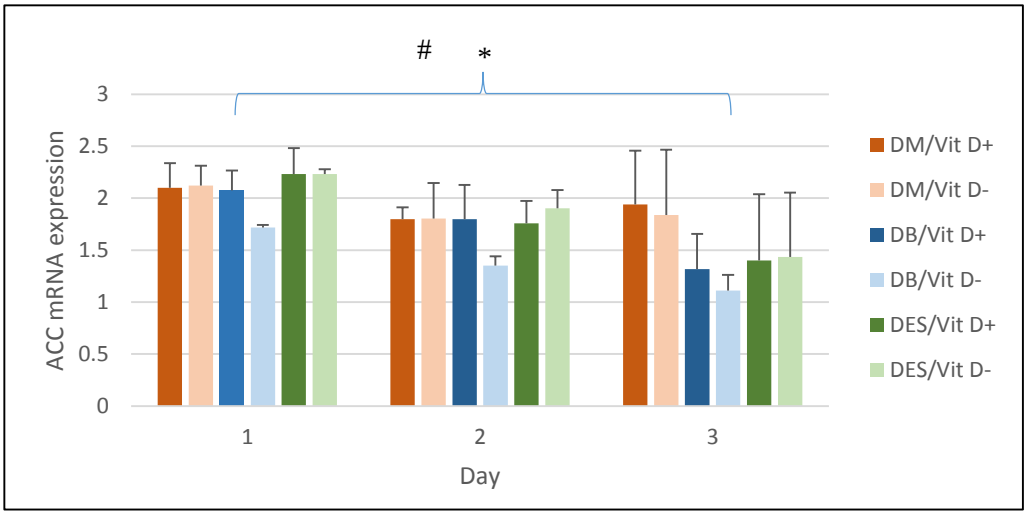
5.2.4.3.4.1 Effects of days of treatment with dbcAMP and Des (1-3) IGF-1 on the mRNA expression related to the lipogenesis

For ACC1 mRNA expression, there was a significant effect of time of incubation ($P<0.05$) with ACC gene expression being the lowest on day 3 (Figure 5.31(A)). For FAS mRNA expression, while there was a significant effect of treatment ($P<0.05$), with dbcAMP treated cells having the lowest expression irrespective of the media used or the incubation time, there was also a very highly significant effect of time of incubation ($P<0.001$) with FAS gene expression being the lowest on day 3 (Figure 5.31(B)).

5.2.4.3.4.2 Effects of days of treatment with dbcAMP and Des (1-3) IGF-1 on the mRNA expression related to the lipolysis

There were no interaction effects in ATGL expression. There was only a trend ($P = 0.063$) for an effect of treatment, with dbcAMP treated cells tended to have the lowest expression (Figure 5.32(A)). However, there was a significant effect of treatment for the expression of HSL mRNA ($P<0.05$), which was dbcAMP treatment gave the lowest expression (Figure 5.32(B)).

A)



B)

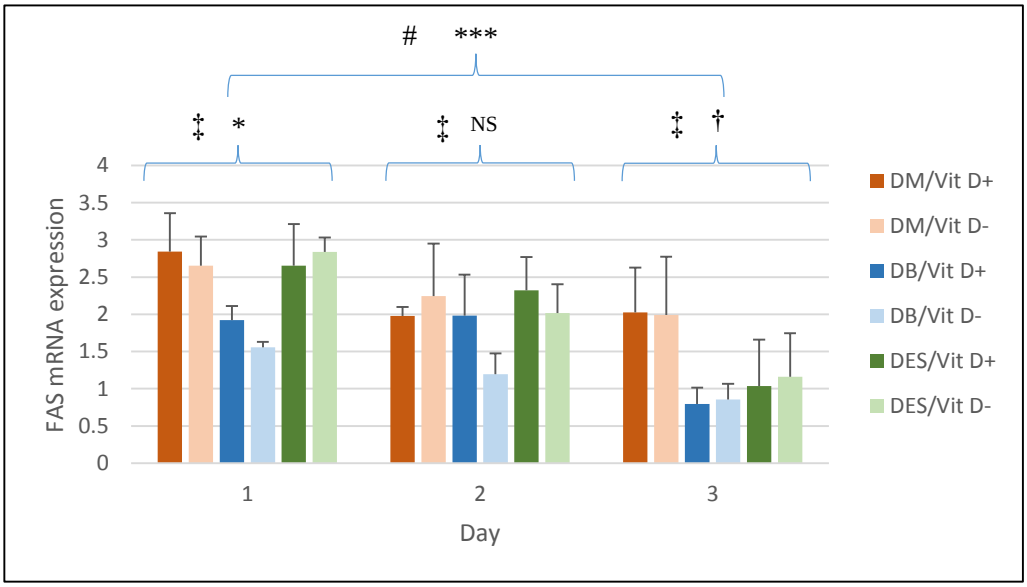
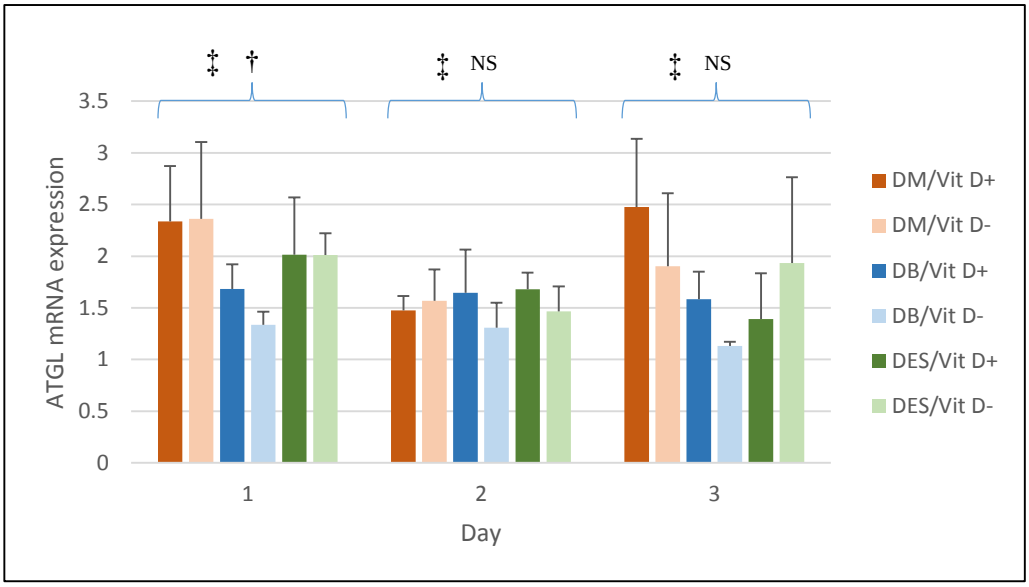


Figure 5.31: Effects of day of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on lipogenic mRNA expression (A) ACC mRNA expression and (B) FAS mRNA expression in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ (indicated as vit D) from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or Des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of time of incubation (Day 1 vs Day 2 vs Day 3).
‡ indicates effect of treatments (DM vs DB vs DES).
*means significant different at $P<0.05$.
***means very highly significant different at $P<0.001$.
† means trend at $P<0.1$.
NS means no significant different.

A)



B)

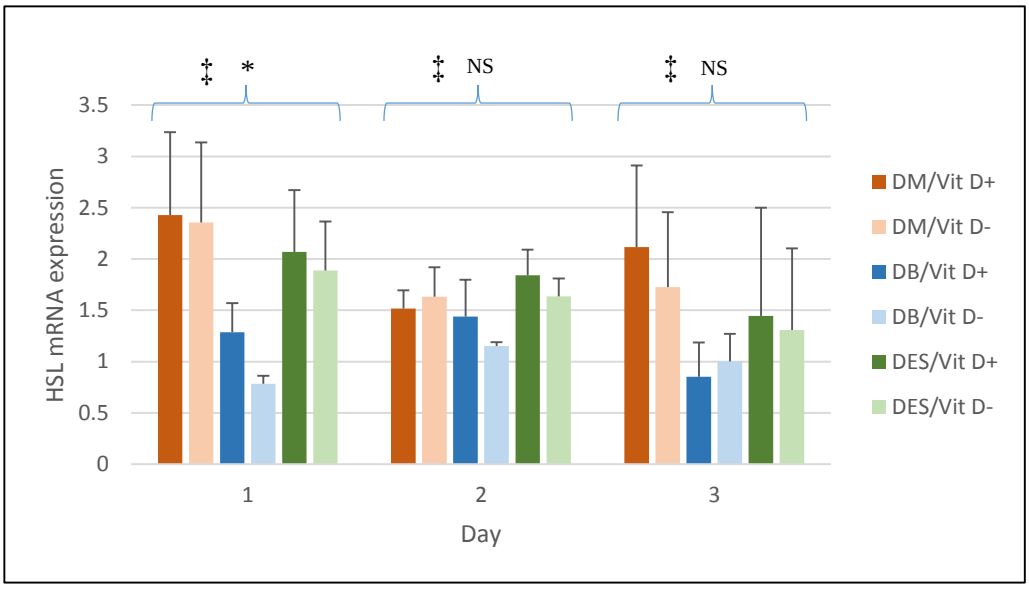


Figure 5.32: Effects of day of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on lipolytic mRNA expression (A) ATGL mRNA expression and (B) HSL mRNA expression in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ (indicated as vit D) from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or Des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
‡ indicates effect of treatments (DM vs DB vs DES).
*means significant different at $P<0.05$.
† means trend at $P<0.1$.
NS means no significant different.

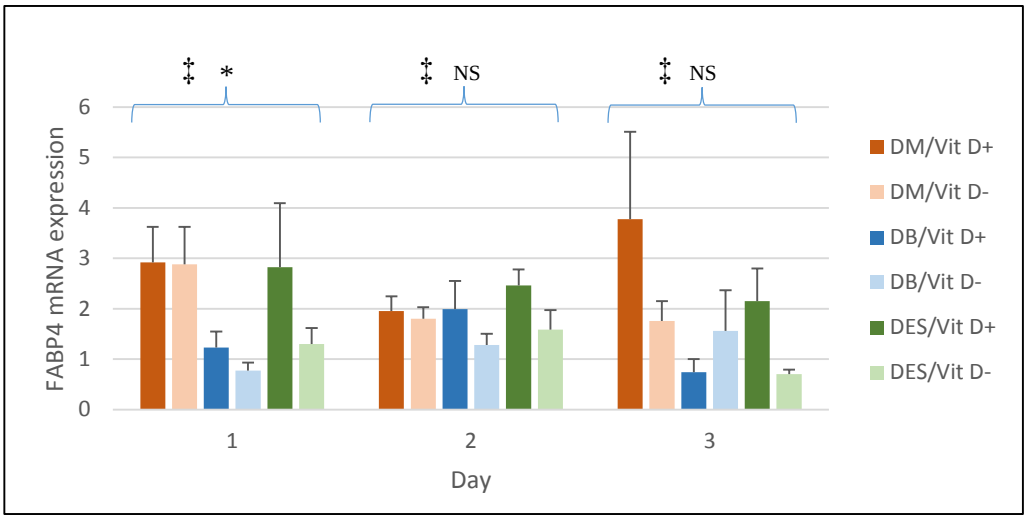
5.2.4.3.4.3 Effects of days of treatment with dbcAMP and Des (1-3) IGF-1 on the mRNA expression related to the adipocyte transcription factors

The FABP4 mRNA expression was significantly reduced ($P<0.05$) in the dbcAMP treated cells, while the expression was significantly higher ($P<0.05$) in cells preincubated with $1,25(OH)_2D_3$, irrespective of treatment (Figure 5.33(A)).

For the expression of PPARγ mRNA, there was an interaction between treatment and media ($P<0.05$) with cells treated with dbcAMP and media without preincubation with $1,25(OH)_2D_3$ having the lowest expression, compared with Des (1-3) IGF-1 treatment in the same media, irrespective of time. There was also effect of treatment ($P<0.05$) with the expression of PPARγ mRNA being higher in Des (1-3) IGF-1 treatment compared with dbcAMP treatment, irrespective of the media used. In addition, incubation time also demonstrated a very highly significant effect on the PPARγ mRNA expression ($P<0.001$) with day 2 having the lowest expression (Figure 5.33(B)).

No effects of any factors and any interactions were found for the mRNA expression of CD36 and C/EBPα (Figure 5.34(A) and Figure 5.34(B), respectively).

A)



B)

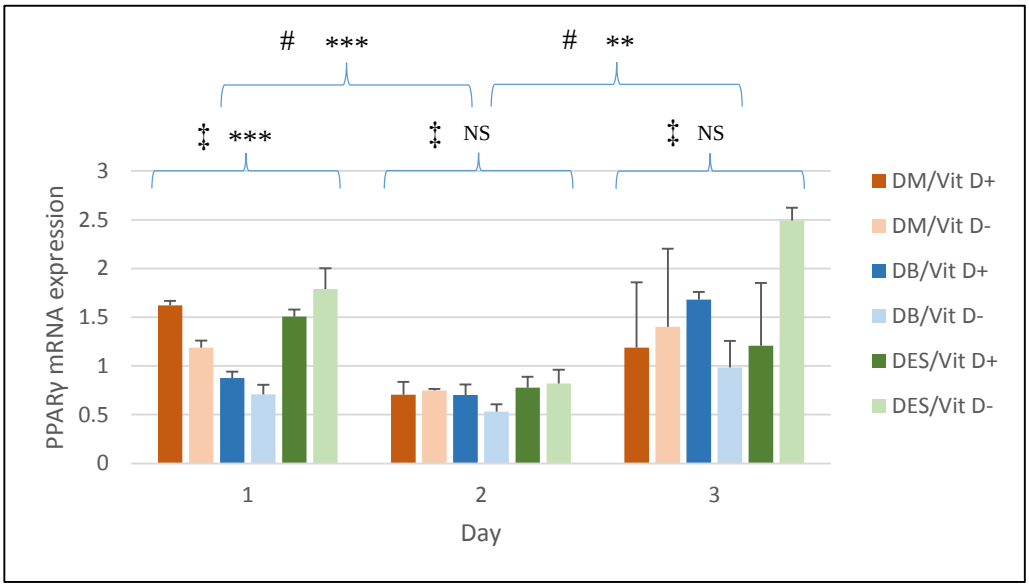
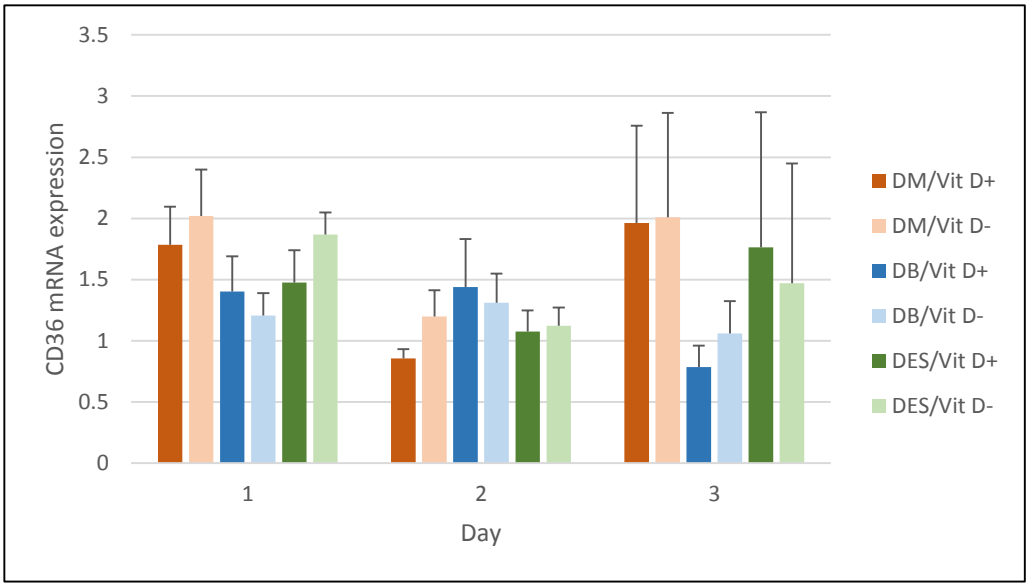


Figure 5.33: Effects of day of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on mRNA expression of adipogenic transcription markers (A) FABP4 mRNA expression and (B) PPARγ mRNA expression in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ (indicated as vit D) from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or Des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of time of incubation (Day 1 vs Day 2 vs Day 3).
‡ indicates effect of treatments (DM vs DB vs DES).
*means significant different at P<0.05.
**means highly significant different at P<0.01.
***means very highly significant different at P<0.001.
NS means no significant different.

A)



B)

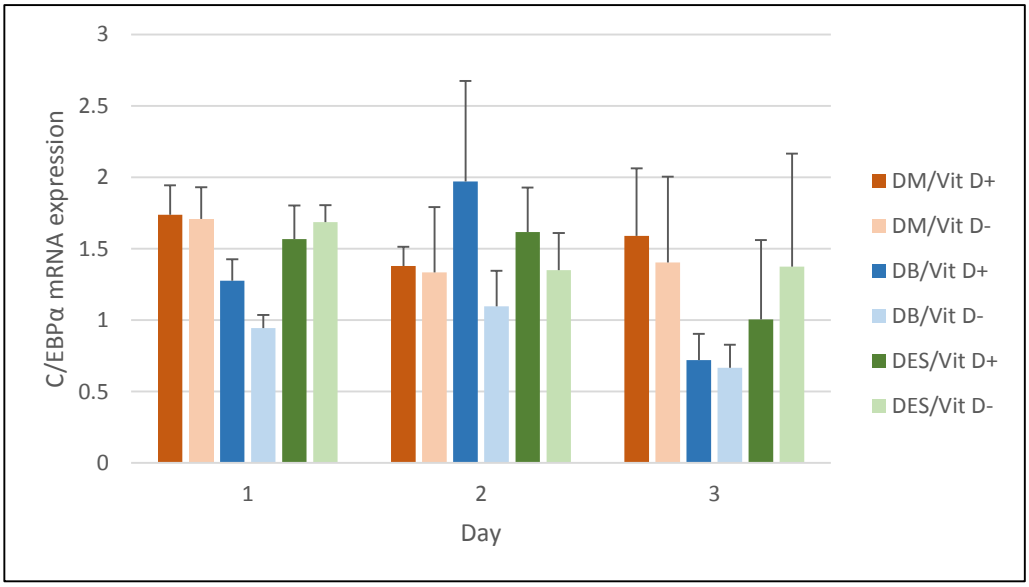


Figure 5.34: Effects of day of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on mRNA expression of adipogenic transcription markers (A) CD36 mRNA expression and (B) C/EBPα mRNA expression in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ (indicated as vit D) from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or Des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
No significant interaction and different between the effects of treatments (DM vs DB vs DES) and time of incubation (Day 1vs Day 2 vs Day 3) in (A) and (B).

5.2.4.3.4.4 Effects of days of treatment with dbcAMP and Des (1-3) IGF-1 on the mRNA expression related to the muscle differentiation marker

There was no interaction among all factors on the expression of creatine kinase mRNAs. However, there was a very highly significant effect of treatment ($P<0.001$) irrespective of time or media, with the treatment of dbcAMP, which elevating the mRNA expression of creatine kinase (Figure 5.35).

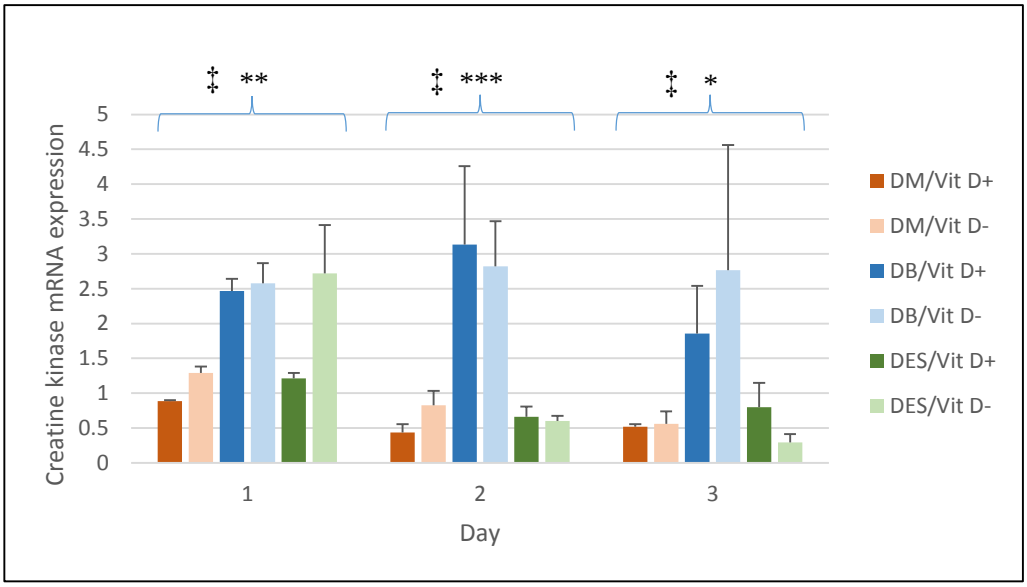


Figure 5.35: Effects of day of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on mRNA expression of muscle differentiation marker, creatine kinase mRNA expression in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ (indicated as vit D) from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or Des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
‡ indicates effect of treatments (DM vs DB vs DES).
*means significant different at $P<0.05$.
**means highly significant different at $P<0.01$.
***means very highly significant different at $P<0.001$.

5.2.4.3.4.5 Effects of days of treatment with dbcAMP and Des (1-3) IGF-1 on the mRNA expression related to the myogenic regulatory factors

For the Myf5 mRNA expression, there were no interaction effects. There was a very highly significant effect of time of incubation ($P<0.001$), which the expression was very highly significantly reduced between day 1 and 2, whilst there was no effect of treatments or the media (Figure 5.36). The similar effect also can be seen on the expression of the MyoD, which was very highly significantly reduced ($P<0.001$) between day 1 and day 2 after treatment (Figure 5.37(A)). For the expression of myogenin, there was a significant interaction between time and treatment ($P<0.05$) with the dbcAMP incubated cells having the highest expression at day 1 (Figure 5.37(B)). There was also a very highly significant ($P<0.001$) decreased in myogenin mRNA expression over the 3 days, similar to that seen for Myf5 and MyoD.

5.2.4.3.4.6 Effects of days of treatment with dbcAMP and Des (1-3) IGF-1 on the mRNA expression related to the myofibrillar protein markers

For MyHCI mRNA expression there was a significant interaction between time and treatment ($P<0.05$) for MyHCI mRNA expression. There was a very highly significant effect of time of incubation ($P<0.001$) with expression of MyHCI decreasing at day 3. The expression also showed a trend ($P = 0.053$) of the effect of the treatment, which dbcAMP treated cells showed high expression compared with other treatments (Figure 5.38(A)). The expression of MyHCIIa mRNA expression had no interactions between the factors but there was a significant effect of time ($P<0.05$), which significantly downregulated at day 3 (Figure 5.38(B)). As for MyHCIIx mRNA expression, there was also no indication of an interaction but expression also significantly downregulated ($P<0.05$) at day 3 irrespective of treatment or media (Figure 5.39(A)). However, for the expression of MyHCIIb, there was a trend of interaction ($P = 0.086$) between time and treatment, which the treatment with dbcAMP tended to increase at day 2 after treatment. There was a very highly significant effect ($P<0.001$) of treatment on MyHCIIb mRNA, irrespective of time or preincubation with $1,25(OH)_2D_3$, as

the expression being very highly significantly upregulated by dbcAMP (Figure 5.39(B)).

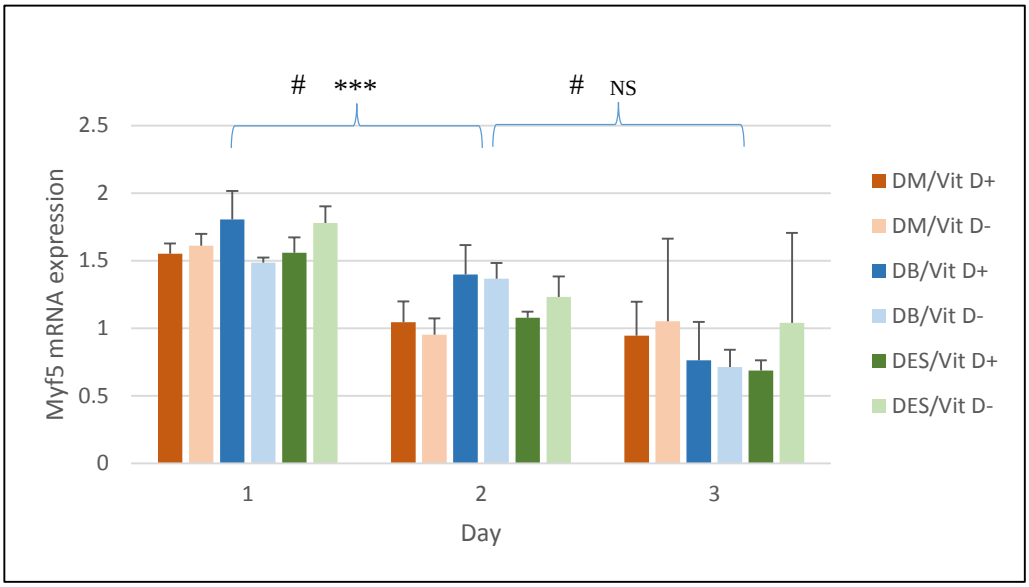
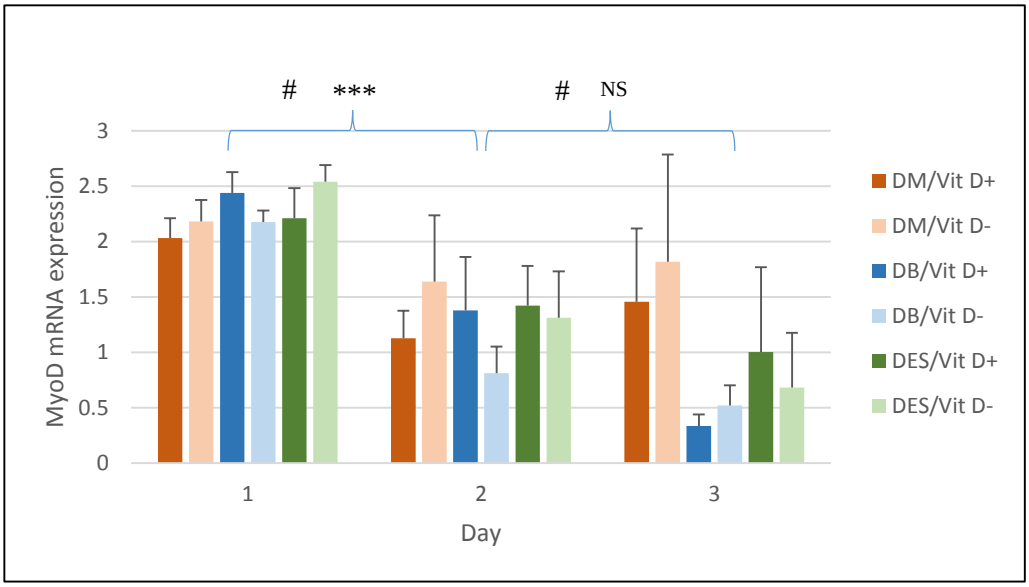


Figure 5.36: Effects of day of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on mRNA expression of myogenic regulatory factor, Myf5 mRNA expression in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ (indicated as vit D) from the point of 70% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or Des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of time of incubation (Day 1 vs Day 2 vs Day 3).
***means very highly significant different at P<0.001.
NS means no significant different.

A)



B)

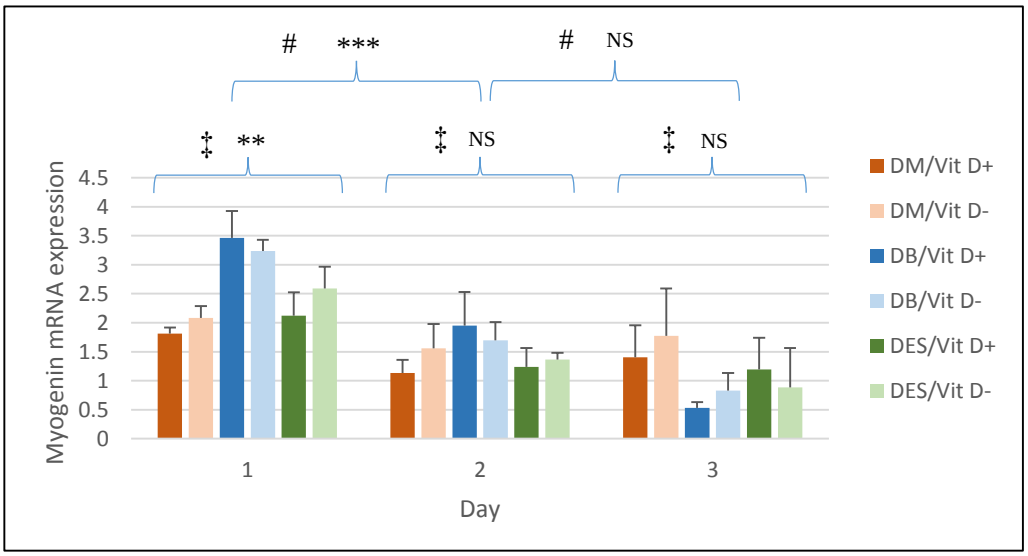
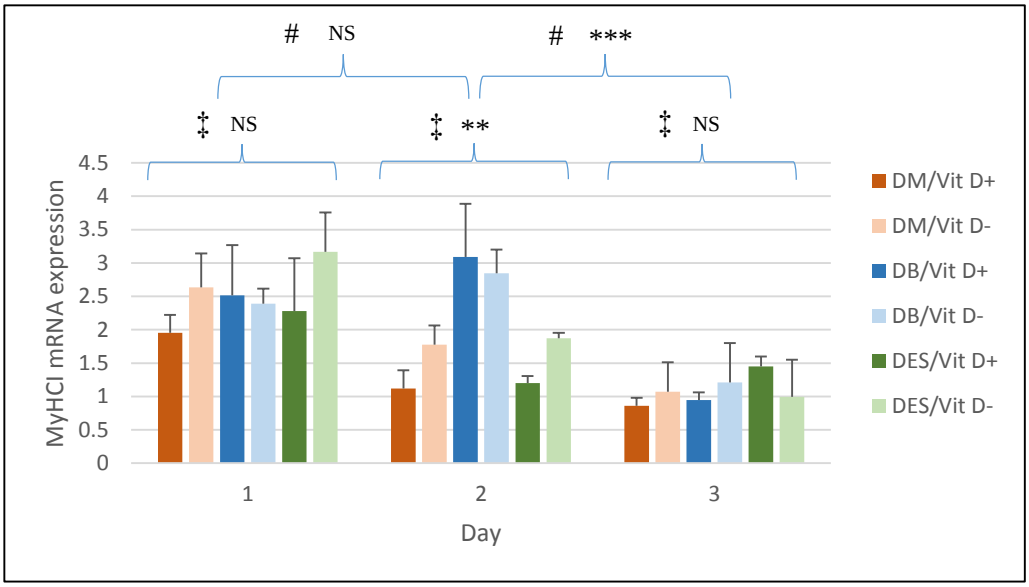


Figure 5.37: Effects of day of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on mRNA expression of myogenic regulatory factor (A) MyoD and (B) myogenin mRNA expression in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ (indicated as vit D) from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or Des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of time of incubation (Day 1 vs Day 2 vs Day 3).
‡ indicates effect of treatments (DM vs DB vs DES).
**means highly significant different at P<0.01.
***means very highly significant different at P<0.001.
NS means no significant different.

A)



B)

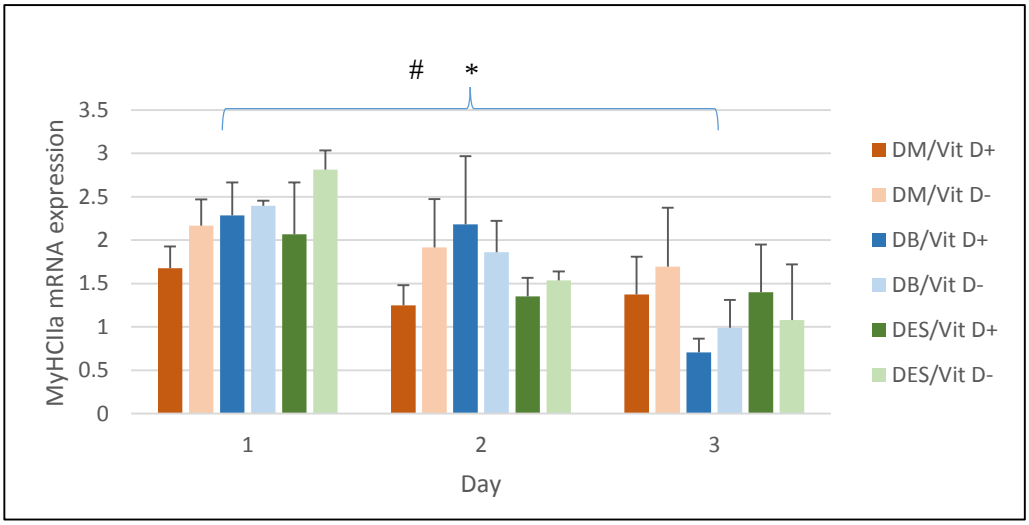
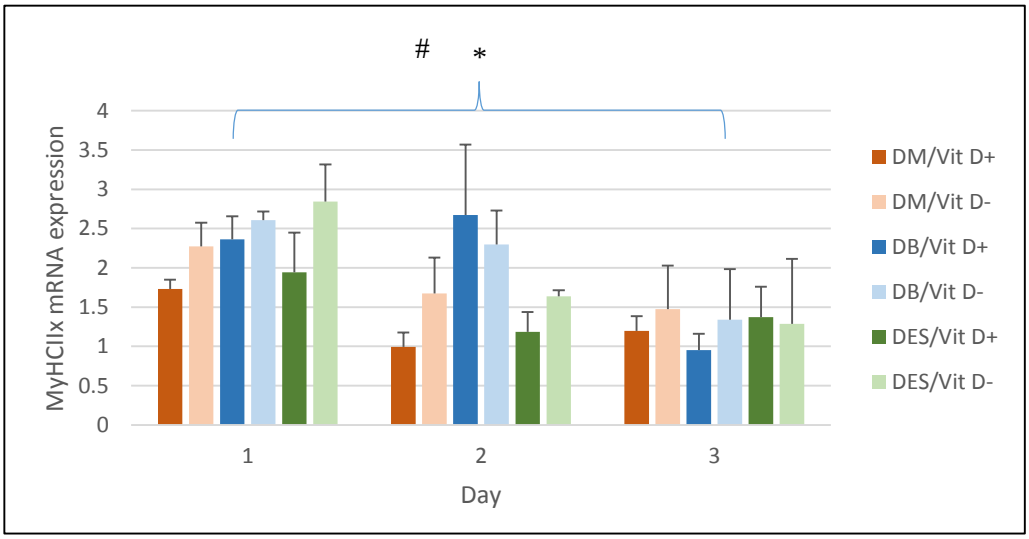


Figure 5.38: Effects of day of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on mRNA expression of myofibrillar protein markers (A) MyHCI mRNA expression and (B) MyHCIIa mRNA expression in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ (indicated as vit D) from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or Des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of time of incubation (Day 1 vs Day 2 vs Day 3).
‡ indicates effect of treatments (DM vs DB vs DES).
*means significant different at P<0.05.
**means highly significant different at P<0.01.
***means very highly significant different at P<0.001.
NS means no significant different.

A)



B)

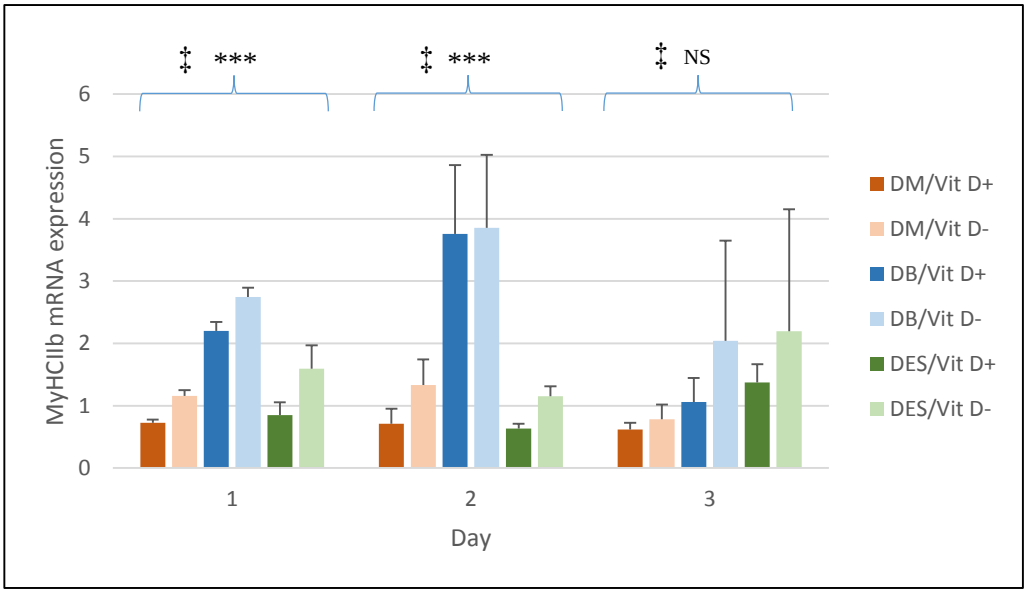


Figure 5.39: Effects of day of treatment with either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on mRNA expression of myofibrillar protein markers (A) MyHCIIx mRNA expression and (B) MyHCIIb mRNA expression in C2C12 cells. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ (indicated as vit D) from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with DMSO, dbcAMP or Des-IGF-1, which have been preincubated with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with n=3 per treatment group. The ANOVA was used for statistical comparison, and subsequent significant value were determined using Bonferroni's test. The error bars are ± SEM.
# indicates effect of time of incubation (Day 1 vs Day 2 vs Day 3).
‡ indicates effect of treatments (DM vs DB vs DES).
*means significant different at P<0.05.
***means very highly significant different at P<0.001.
NS means no significant different.

Table 5.2: Summary for the significant value for the effects and interaction on the expression of adipogenic-related genes

Type	Gene name	Effects / Interaction (P value)						
		Day	**Treatment**	**[Vit D]**	**Day x Treatment**	**Day x [Vit D]**	**Treatment x [Vit D]**	**Day x Treatment x [Vit D]**
Lipogenic	ACC1	<0.05	NS	NS	NS	NS	NS	NS
	FAS	<0.001	<0.05	NS	NS	NS	NS	NS
Lipolytic	ATGL	NS	0.063	NS	NS	NS	NS	NS
	HSL	NS	<0.05	NS	NS	NS	NS	NS
Adipocyte Transcription Factors	PPARγ	<0.001	<0.05	NS	NS	NS	<0.05	NS
	CD36	NS	NS	NS	NS	NS	NS	NS
	CEBPα	NS	NS	NS	NS	NS	NS	NS
	FABP4	NS	<0.05	<0.05	NS	NS	NS	NS

Means significant effect of the factor and/or interaction at P<0.05.
Means very highly significant effect of the factor and/or interaction at P<0.001.
Means trend of the factor and interaction at P<0.1.

NS = not significant

[Vit D] = status with or without preincubation with $1,25(OH)_2D_3$

Table 5.3: Summary for the significant value for the effects and interactions on the expression of myogenic-related genes

Type	Gene name	Effects / Interaction (P value)						
		Day	**Treatment**	**[Vit D]**	**Day x Treatment**	**Day x [Vit D]**	**Treatment x [Vit D]**	**Day x Treatment x [Vit D]**
Muscle Diff. Marker	Creatine Kinase	NS	<0.001	NS	NS	NS	NS	NS
Myogenic Regulatory Factors	Myf5	<0.001	NS	NS	NS	NS	NS	NS
	MyoD	<0.001	NS	NS	NS	NS	NS	NS
	Myogenin	<0.001	NS	NS	<0.05	NS	NS	NS
Myofibrillar Markers	MyHCI	<0.001	0.053	NS	<0.05	NS	NS	NS
	MyHCIIa	<0.05	NS	NS	NS	NS	NS	NS
	MyHCIIb	NS	<0.001	NS	0.086	NS	NS	NS
	MyHCIIx	<0.05	NS	NS	NS	NS	NS	NS

Means significant effect of the factor and/or interaction at P<0.05.
Means very highly significant effect of the factor and/or interaction at P<0.001.
Means trend of the factor and/or interaction at P<0.1.
NS = not significant
[Vit D] = status with or without preincubation with $1,25(OH)_2D_3$

5.2.4.4 Summary

Treatment of dbcAMP and Des (1-3) IGF-1, which mimics the action of BA and IGF-1 (GH indirectly) respectively, demonstrated that both agents affect the lipid metabolism in the differentiating C2C12 in adipogenic media, while the expression of certain genes were strongly induced with prolong treatment. The dbcAMP predominantly appeared to reduce lipogenic activity, while reducing lipid turnover in the cells, and Des (1-3) IGF-1 was demonstrated to induce adipogenesis, while the cells both retaining the 'true' muscle characteristics, by the effect of dbcAMP by expressing myogenic-related factors.

5.3 DISCUSSION

5.3.1 C2C12 as an intramuscular development model

In this experiment, the mouse myoblast cell lines, C2C12, were exposed to adipogenic media, consisting of insulin, dexamethasone (DEX) and isobutylmethylxanthine (IBMX), to promote adipogenesis. This media is normally used to induce preadipocyte to adipocyte differentiation in 3T3 preadipocytes. The formation of lipid droplets (visualized using the Oil Red O staining method), showed that the media had induced the transdifferentiation of the C2C12 cells into mature adipocytes, whilst they also retained their capacity to differentiate into myotubes (Ryan et al., 2013). The induction of adipogenesis of 3T3-L1 cell lines (fibroblast cell line) with adipogenic media have been reported by Katafuchi et al. (2010). In these cells, the insulin in the media is involved in the activation of ERK and Akt/PKB pathways, and stimulating glucose uptake, which is stored as triacylglycerol (Moseti et al., 2016). The DEX inhibits the expression of Pref-1, which is an inhibitor of adipogenesis, whilst the IBMX increases intracellular cAMP and thereby protein kinase A (PKA) activity which is required for transcriptional activation of peroxisome proliferator-activated receptor (PPAR)γ and adipogenic gene expression, by inhibiting the activity of phosphodiesterases (Scott et al., 2011; Katafuchi et al., 2010). DEX and IBMX induce the CCAAT/enhancer binding protein, C/EBPδ and C/EBPβ expressions, respectively, and, to form heterodimerization of C/EBP)β/δ heterodimer, which subsequently activates PPARγ and C/EBPα to stimulate adipogenesis and inhibit myogenesis (Moseti et al., 2016; Farmer, 2006; Sun et al., 2003; Yeow et al., 2001; Hu et al., 1995). The C2C12 myogenic cells transdifferentiation to adipocytes is also influenced by the rosiglitazone treatment. Rosiglitazone upregulates the transcription factor PPARγ, by the expression of domain negative (DN) MKK3 (Yeow et al., 2001).

The C2C12 myoblasts cell line, which derived from the mouse skeletal muscle, originally subcloned from C2 myoblasts (Yaffe and Saxel, 1977) provides a good model for the *in vitro* study of myogenesis (Burattini et al., 2004). However, in this study, we used this cell line for the intramuscular development

model by transdifferentiation of myoblasts to adipoblasts by incubation in specific differentiation media (refer as adipogenic media) as discussed below. The chronic exposure of the C2C12 myoblasts with several treatments such as long-chain fatty acids (LCFAs) and thiazolidinediones compound pioglitazone prevent the formation of myotubes, induce formation of lipid-containing cells or lipid droplets accumulation, inhibition of creatine kinase activity, myogenin and α-actin mRNAs downregulation, increased glycerophosphate dehydrogenase (GPDH) activity, and upregulation of fatty acid transporter (FAT) and hormone sensitive lipase (HSL) mRNAs (Grimaldi et al., 1997) suggesting the trans-differentiation has taken place. A study by Singh et al. (2007) induced the trans-differentiation process with adipogenic media supplemented with thiazolidinediones as a ligand for PPARγ, activate the adipogenic program, indicated by the accumulation of lipid droplets in the cellular matrix and increase in adipoblast formation. This approach was similar with our study, which used rosiglitazone, another PPARγ-ligand instead of thiazolidinediones.

5.3.2 Effects of differentiation medium, myogenic and adipogenic media on the gene expressions

The present experiment demonstrated the differentiation of the cell were affected by different growth media. The growth media is usually supplemented with the fetal bovine serum (FBS), which is one of the factor for the continuation of cell proliferation. The differentiation will be triggered by switching the higher serum percentage to the lower serum percentage (2-10%) at the confluency point (Portier et al., 1999). Portier and colleagues also proved that the use of the serum substitute, Ultroser G and rat brain extract, resulted in increase in ten degrees of myotubes maturation, and contributed to the higher differentiation grade of the cells (Portier et al., 1999). Lawson and Purslow (2000) also demonstrated that serum-free medium resulted in increased myotubes formation and increase creatine phosphokinase (CPK) activity in C2C12 cells. This suggested that reduced serum percentages will reduced the number of factors for proliferation, and consequently induced differentiation and this probably applies to the use of differentiation media. The commonly used 2% horse serum and 10% FBS in the myogenic and adipogenic media respectively, lead to differing development and

resulting phenotype of the cell. The effect of adipogenic media is to stimulate C2C12 transdifferentiation into adipocytes, characterized by the presence of lipid droplets accumulation in the cells differentiated in adipogenic media, which cannot be seen in the cells differentiated in the myogenic media. The presence of fusing of myoblasts and myotubes formation in the cells of both media was indicated by there being no significant difference in the quantity of creatine kinase gene expression. Likewise, for myogenin gene expression, which was not significant between both media. Myogenin is usually expressed at terminal differentiation stage (Burattini et al., 2004). As would be expected, as the cell differentiated gene expression of the MyHC isoforms increased in myogenic media. The exposure of C2C12 cells to the cocktail of FBS, insulin, IBMX and DEX in the adipogenic media is associated with the stimulation of C/EBPβ and C/EBPδ expression, which has been reported to lead to the expression of PPARγ and C/EBPα (Singh et al., 2007; Hu et al., 1995). Also present in the adipogenic media was rosiglitazone, one of the thiazolidinediones family, which is an activator for the PPARγ expression (Benvenuti et al., 2007; Yeow et al., 2001), as well as the adiponectin (Farmer, 2005). PPARγ also have been shown to be essential for preadipocyte differentiation into mature adipocytes (Rosen et al., 2000). The upregulation of FABP4 also stimulated by the adipogenic program that was activated during the apparent transdifferentiation of C2C12 cells into adipocytes in the adipogenic media is an indicator that adipocytes have formed as its expression is predominantly found in in fat cells (Haunerland and Spener, 2004; Taylor-Jones et al., 2002). This might be expected as insulin and thiazolidinediones which activate the expression of PPARγ and this transcription factor is involved in the transcriptional regulation of FABP4 as peroxisome-proliferator response element (PPRE) is present in FABP4 gene promoter (Smathers and Petersen, 2011; Krusinova and Pelikanova, 2008; Haunerfield and Spener, 2004; Schachtrup et al., 2004).

5.3.3 Dose-dependent effects of 1,25(OH)$_2$ vitamin D$_3$ in on expression of myogenesis- or adipogenesis-related genes in differentiating C2C12 cells

In this dose-dependent experiment, both concentrations of 1,25(OH)$_2$D$_3$ did not affect the myogenesis- or adipogenesis-related gene expression. These concentrations were equivalent to a physiologically low dose, reflective of a D3 deficiency, and had been previously described as the deposition of lipid droplets in transdifferentiating C2C12 cells (Ryan et al., 2013). However, there were strong effects of incubation media. As observed in the previous experiment, there was increased expression in genes associated with adipogenic differentiation. The adipogenic media increased the expression of FABP4 whilst there was no detectable expression in myogenic media. This further suggested the FABP4 is a marker for the adipogenesis which reflects its role in lipid transports and accumulations (Moseti et al., 2016; Zimmerman et al., 2002). Its expression was strongly associated with deposition of the lipid droplets as visualized by the Oil Red O staining, which only occur in the cells treated in adipogenic media. This was in contrast to the PPARγ which was expressed in the cells treated with myogenic media, suggesting that PPARγ probably plays a role in during myogenesis (Hunter et al., 2001). During the induction in the myogenic program (in myogenic media), the C2C12 differentiation was demonstrated to be regulated by mammalian target of rapamycin (mTOR) mechanism, which was also reported to involve in the PPARγ stimulation (Blanchard et al., 2012; Erbay and Chen, 2001). Altered PPARγ expression, either by overexpression or deletion, affect the myotube formation and has been described as reducing myogenic markers such as myogenin and creatine kinase expressions and activities, suggesting PPARγ has a role as a myogenic transcriptional regulator (Singh et al., 2006).

Surprisingly, the creatine kinase and myogenin expressions were induced by adipogenic media, which was not in line with our previous findings. One probability is the effects of DMSO, which have been demonstrated to inhibit myogenesis, by delaying the myotube formation, altering the expression of myogenin and reducing creatine kinase activity during C2C12 differentiation (Orzechowski et al., 2001). However, DMSO was also present in adipogenic

media, suggesting possible mechanism and interaction between the DMSO and component in adipogenic media, which induced myogenic marker expressions, which needs further elucidation. In addition, we also might suggest the measurement could be done at three time points, such as at day 2, day 4 and day 6, which might be useful to determine the trends or patterns of those gene expressions to check for any discrepancies. From this suggestion, we might hypothesize the upregulation of creatine kinase and myogenin gene expressions at day 2 in myogenic media compared with adipogenic media at the same time point.

5.3.4 Effects of day of treatment with dbcAMP and Des (1-3) IGF-1 following preincubation with $1,25(OH)_2D_3$ in adipogenic media on myogenesis- and adipogenesis- related mRNA expression

The current study demonstrated the effects of dbcAMP (DB) and Des (1-3) IGF-1 (DES) on myogenesis and adipogenesis in C2C12 myoblast cells, including the interaction with the effects of preincubation of the C2C12 cells in adipogenic media which included $1,25(OH)_2D_3$ supplementation. During myogenesis, there are morphological changes as cells move from myoblasts and then fuse to form multi-nucleated myotubes. In the presence of an adipogenic induction, the myotubes still developed but this was accompanied by the formation of the lipid droplets, suggesting that there was both myogenesis and transdifferentiation from myoblast to "adipo-like" cells occurred in the culture.

During preincubation (without dbcAMP and Des (1-3) IGF-1), the lipid droplets accumulation in the treated cells both with DMSO or $1,25(OH)_2D_3$ were increased at day 6 irrespective to the treatment, suggesting an increased rate of adipogenesis. Dose-dependent study of $1,25(OH)_2D_3$ by Ryan et al. (2013), demonstrated that the lipid droplets were increased by lowest concentration (10^{-13} M), relative to DMSO (control), whilst this was not significant at 10^{-11} M. As 10^{-12} M $1,25(OH)_2D_3$ was used in this experiment, this might have suggested that it still not enough to induce a significant lipid droplets accumulation. Associated with lipid droplet formation, there was increased FABP4 expression at day 6, there being no difference between DMSO or $1,25(OH)_2D_3$ incubation suggesting

transdifferentiation into adipo-like cells, as demonstrated by recent study by Zhang et al. (2017). The increased lipid droplets in this cell culture system could be analogous to an increase of IMF deposition *in vivo*. This suggestion is further strengthened by the associated observation that FABP4 gene expression was induced in the C2C12 exposed to adipogenic as FABP4 expression has been reported to positively correlate with IMF (Jurie et al., 2007). For example, higher IMF in LD muscle of Japanese black steers was associated with the higher FABP4 expression (Wang et al., 2005). Furthermore, the expression of FABP4 in the muscle also indicative for the adipocyte differentiation as it expressed in the adipocytes, but not in the muscle fibres (Jurie et al., 2007).

The treatment with dbcAMP or Des (1-3) IGF-1, resulted in significant changes in the expression of several genes, along with the reduction in the lipid droplets deposition in virtually all the treatment groups. The reduction of FAS by dbcAMP, indicated a reduction in lipogenesis. The HSL expression was also reduced in dbcAMP treatment, showing its strong effect in reducing lipid turnover in the cells, as indicated by the observation that ATGL gene expression tended to be reduced as well. BA action, which is mediated through cAMP-dependent protein kinase, is also reported to reduce lipogenesis in porcine fat explants (Peterla and Scanes, 1990). This data suggested the reduction of lipid turnover might also associated with the reduction of lipogenesis by dbcAMP. This was also associated with the decreased of lipogenic ACC and FAS expressions after 3 days treatment, while no effect on lipolytic ATGL and HSL genes, suggesting reduced lipogenic capacity after prolonged treatment.

Adipogenesis has been demonstrated to be regulated by Des (1-3) IGF-1, which increased the expression of the key adipogenic gene, PPARγ. Des (1-3) IGF-1, which is one of the IGF-1 variants, is the mediator in the GH response (Ballard et al., 1996). GH has been demonstrated to increase PPARγ expression in the 3T3-L1 preadipocytes via the JAK/STAT pathway (Kawai et al., 2007). Another study by Kim et al. (2010) demonstrated the activation of PPARγ was induced by Akt signalling, which was apparently activated by IGF-1 during adipocyte differentiation as the activation was blocked by an Akt/PI3K inhibitor and knockdown of Akt1 (as explained in Section 2.4.3). The induction of

PPARγ would suggest the increase in preadipocyte differentiation toward mature adipocyte, which may involve in the adipo-like cells transdifferentiation from myoblasts. This effect was not associated with the reduction in lipid droplets deposition, thus, it was unclear as PPARγ was reported to regulate the perilipin-1 gene (Plin1) expression, a lipid droplets-coating protein, required for the formation of lipid droplets (Arimura et al., 2004; Dalen et al., 2004), suggesting the lipid droplets formation might be due to reduced posttranslational activity of PPARγ, which was not measured in this study. According to Hu et al. (1995), ectopic expression of PPARγ did not induce the lipid droplets formation, unless the effect was synergized with C/EBPα. The expression of C/EBPα was not affected in this study, and this suggest the reduction in lipid droplets was not due to the synergistic effect between both genes. The number of lipid droplets in Des (1-3) IGF-1 treated cells were not as high as in the DMSO-treated cells. This may due to deposition of lipid being strongly induced by the lipogenic gene such as FAS and/or FABP, whilst the effect of PPARγ strongly induced adipogenesis only, and these genes playing slightly different roles in lipid metabolism.

In general, the adipogenic media induced the expression of FABP4 in all groups. It is categorized as adipocyte marker due to it specific expression in adipose tissue. Thus, the reduced of FABP4 mRNA expression after dbcAMP treatment, suggested reduced adipogenesis. As described above, *in vivo*, the expression of FABP4 mRNA indicated the IMF formation, and this suggest the role of dbcAMP in reduced IMF deposition, which was associated with reduction in lipid droplets formation (Jurie et al., 2007). Although other study by Okuda et al. (1989) demonstrated the p-aminophenol, which has similar structural characteristics with dbcAMP, induced lipolysis in rat adipocytes, and suggested dbcAMP as lipolytic agents (Mills and Liu, 1990). It already suggested above that dbcAMP strongly reduced lipid turnover, while the significant reduction in the FABP4 gene expression might also suggest dbcAMP, and therefore agents such as BA which stimulate cAMP production, play a role in reduction in IMF deposition. In addition, the FABP4 transcript expression was increased by the $1,25(OH)_2D_3$ incubation, which was in line with the observations of Ryan et al. (2013), demonstrated the dose of $1,25(OH)_2D_3$ between 10^{-13} M to 10^{-11} M (which considered as low physiological concentration) increased FABP4

expression compared with other doses. Later, it was reported that the high responsiveness of FABP4 was observed towards $1,25(OH)_2D_3$, and the expression was inhibited by all concentrations (10^{-11} M - 10^{-7} M) (Ji et al., 2015). However, we still might suggest that the dose used in this experiment (10^{-12} M) was outside the range, and probably would stimulate FABP4 expression. However, this might not be associated with the deposition of lipid droplets (as described above), due to interaction of $1,25(OH)_2D_3$ with the treatments, which still need to be determined further.

In regards to myogenesis, the effect of dbcAMP was strong than the other treatments in proliferation, differentiation or myofibril development. The creatine kinase (CK) expression was increased following dbcAMP treatment, indicating stimulation of cell differentiation. This effect was similar to the BA treatment (clenbuterol) on neonatal rat muscle cell lines, which stimulated fusion and formation of myotubes, indicated by increased CK activity (McMillan et al., 1992). This finding may suggest potential role of dbcAMP, and agents which stimulate cAMP production, such as BA, in enhancing myogenesis, and further indicates that muscle hypertrophic effect *in vivo* is mediated by cAMP-dependent signalling pathways.

The myogenic regulatory factors (MRFs), which are Myf5, MyoD, Myogenin, and MRF4 control the regulation and mechanism of the myogenic development from progenitor cell commitment to myogenesis through to the fusion of mononucleated myoblasts to form myotubes (Yan et al., 2013; Sabourin and Rudnicki, 2000). In current study, dbcAMP increased myogenin mRNA expression at day 1 after treatment, and reduced it afterwards, suggesting the accelerated stimulation of myoblast differentiation to form myotubes. This effect reflects the changes in creatine kinase gene expression which is indicative myotube differentiation markers (as described above).

The changes in myofibrillar markers were also observed by the effect of dbcAMP compared with other agents. The dbcAMP tended to increase MyHCI and significantly stimulate MyHCIIb expression at day 2. Study by Brown et al. (2011) demonstrated, during myogenesis of the untreated cells, the MyHCI

expression peaked at day 4 while MyHCIIb peaked at day 8 after differentiation, which is in line with current study (cells have been differentiated for 8 days). However, the greatest effect of dbcAMP was on increasing MyHCIIb gene expression. This might be due to MyHCIIb being the predominant myosin heavy chain isoform in rodent muscle, and is highly responsive toward treatment/stimuli (Harrison et al., 2011).

When we considered the effect of day as a factor, there was generally a reduction in gene expression after the treatment including the lipogenic genes, as well as MRFs and myofibrillar markers. This might be due to the reduction of cells, possibly due to cell death, particularly apoptosis, as there was reduction of RNA quantity towards the end of the experiment. However, we still cannot conclusively make this assumption as DNA quantity was not assessed in this experiment, which could have given an insight regarding the interaction between days of treatment and reduced in expression of certain genes.

5.4 CONCLUSION

During differentiation with adipogenic media, the C2C12 cells expressed genes associated with adipogenesis, including adipocyte markers such as FABP4. This strongly suggests there was transdifferentiation of myogenic cells into adipogenic cells, whilst the cells retained the muscle differentiating state as there was still gene expression of myogenic differentiation, for example creatine kinase gene expression. Thus, we have established a potential model for examining intramuscular fat development, which appears to consist of muscle and fat cells in one population, thereby exhibiting characteristics found *in vivo* muscle tissues. By utilising this model, the effects of dbcAMP and Des (1-3) IGF-1 were determined. The effect of these agents appeared to reduce lipogenesis, and lipid turnover, as determined by the changes in mRNA expressions, which suggested that both treatments strongly affect the lipid deposition in cells. Therefore this C2C12 mixed cell type culture model could be used as a tool to indicate the potential effects of factors that alter muscle metabolism, thereby indicating their potential effects in muscles *in vivo*. This

has applications in the identification of treatments that may allow the development of lean and healthy meat or understanding the mechanisms for reducing fat deposition in muscles in human, which may have health benefits.

6. GENERAL DISCUSSION

The main hypothesis in this thesis was the metabolic modifiers, beta-agonists (BA) and growth hormone (GH) enhance lean muscle production via manipulation in lipid metabolism, which involves reducing lipogenesis and inducing lipolysis. In this thesis, we determined the effects of agents on lipogenic and lipolytic capacity of BA- and GH-treated sheep in the subcutaneous fat, liver and skeletal muscles (*in vivo*). In addition, we also determined if the changes in the sheep were similar to the effect in a mixed cell population of differentiated C2C12 myotubes, which contained adipocytes, treated with dibutyryl cyclic adenosine monophosphate (dbcAMP) and Des (1-3) insulin-like growth factor-1 (Des (1-3) IGF-1).

Our *in vitro* study utilized the mouse myoblasts, C2C12 cells as a model for the muscle development. The cells incubated with adipogenic media, induced the transdifferentiation of muscle cells into adipocytes, indicated by the accumulation of lipid droplets inside the cells, and demonstrated by the increase in adipocyte markers such as PPARγ and FABP4. However, the cell population retained expression of myogenic factors such as MRFs and creatine kinase, as well as the expression of genes encoding myofibril proteins such as myosin heavy chain. Thus, this model appears to exhibit a mixed population of adipocytes alongside myotubes, corresponding to the intramuscular fat model, as seen in *in vivo* muscles.

6.1 MAJOR FINDINGS

The *in vivo* study examining the effects of BA and GH treatment on sheep, demonstrated the stronger effect of GH than BA on lipid metabolism, with apparent effects on lipogenesis, and adipogenesis, whilst to BA strongly affected muscle hypertrophy, which was reflected in changes in the carcass characteristic. Meanwhile, the *in vitro* study examining the effects of dbcAMP and Des (1-3) IGF-1, which mimic the action of BA and GH, respectively, demonstrated the

strong effect of dbcAMP in affecting lipogenic activity, while expressing myogenic-related factors, while Des (1-3) IGF-1 affected adipogenesis.

6.1.1 Effects of BA

Even over a short period (6 days), according to the carcass characteristics, BA induced muscle hypertrophy. In order to support muscle protein synthesis, which can be measured by the increased of plasma urea concentration, BA appeared to induce direct utilization of amino acids. Metabolomics analysis showed BA significantly reduced plasma essential amino acid concentration, such as histidine and methionine. Likewise, BA numerically (not statistically) reduced the circulating branched-chain amino acids, BCAAs; leucine, isoleucine and valine) concentration, suggesting these amino acids, particularly leucine, are involved in activating protein synthesis (Norton and Layman, 2006). The specificity of BA effect on muscle hypertrophy also has been demonstrated by microarray analysis. The principle finding was gene expression related to the synthetic pathway for the non-essential amino acid, serine was induced (Al-Doski, 2015). However, the metabolomics analysis in the current study showed the plasma serine concentration was not affected by the BA treatment. Thus, this type of observation suggested that the plasma metabolomics analysis could not conclusively indicate of where and how the metabolites were being utilised.

Our *in vitro* study on the C2C12 myogenic cells model demonstrated the treatment of dbcAMP strongly affected the expression of myogenic-related genes rather than adipogenic-related genes. The creatine kinase mRNA expression was increased, indicating BA induced myogenic cell differentiation into myotubes, as similarly demonstrated by McMillan et al. (1992). In addition, dbcAMP also appeared to induce earlier differentiation, as early as 1 day, as indicated by the increased myogenin mRNA expression. In addition, there were effects of dbcAMP on myofibrillar marker gene expression, which also indicated acceleration of differentiation and formation of fibres in myogenic cells. This result also indicates the dbcAMP induced the fibre transition towards fast fibres, which coincide with myotube hypertrophy (Brown et al., 2012). This has been supported by myostatin knockout study in mice, which demonstrated muscle

hypertrophy was associated with increased proportion and hypertrophy of type IIb fibres (Matsakas et al., 2010). In addition, the increased in type IIb fibres transcripts also associated with high muscularity in pigs (Wimmers et al., 2008), which might be associated with muscle hypertrophy.

In terms of lipid metabolism in the *in vivo* sheep study, the BA treatment also increased the plasma glycerol concentration, whilst there were no changes in the NEFA plasma concentrations. Previous studies have shown that BA reduced lipolytic gene expression in muscle (Ferreira et al., 2013). Indeed, the current study showed a trend for a decrease in skeletal muscle ATGL and HSL gene expression suggesting decrease in lipolytic activity. In skeletal lipogenic genes, particularly FAS, had reduced expression. This suggests BA reduced lipid turnover in the muscle. In subcutaneous fat, there was no effect on lipolytic or lipogenic gene expression. However, it must be remembered that the activity of lipolytic enzyme activity, particularly HSL is strongly stimulated by cAMP via post-transcriptional mechanisms which may explain the increase in plasma glycerol concentration. It has been reported that effects of BA on lipolysis tend to be transient lasting 3-4 days (Ferreira et al., 2013) and enhance after 6 days, perhaps only the increase in plasma glycerol can be observed. The potential effects of BA on reducing of lipid content of tissues was demonstrated by the treatment of BA-mimicking agent, dbcAMP on myogenic C2C12 cells model. Upon dbcAMP treatment, FAS mRNA expression was strongly reduced while ATGL was also reduced, thereby reflecting the effect of BA *in vivo* on muscle lipid turnover. The reduction in lipid turnover indicates the potential reduction of synthetic capacity in muscles, which could contribute to the reduction of intramuscular fat (IMF) stimulated by BA. A reduction in this type of fat in muscle would be expected to have the effect of reducing meat quality, particularly the aspects of juiciness and flavour. However, the IMF could be considered as unhealthy and thus, the usage of BA in meat production could be beneficial in producing leaner meat which is considered to be healthy (Dunshea et al., 2005).

The effects of BA treatment *in vivo* according to the target organs were summarized in Figure 6.1.

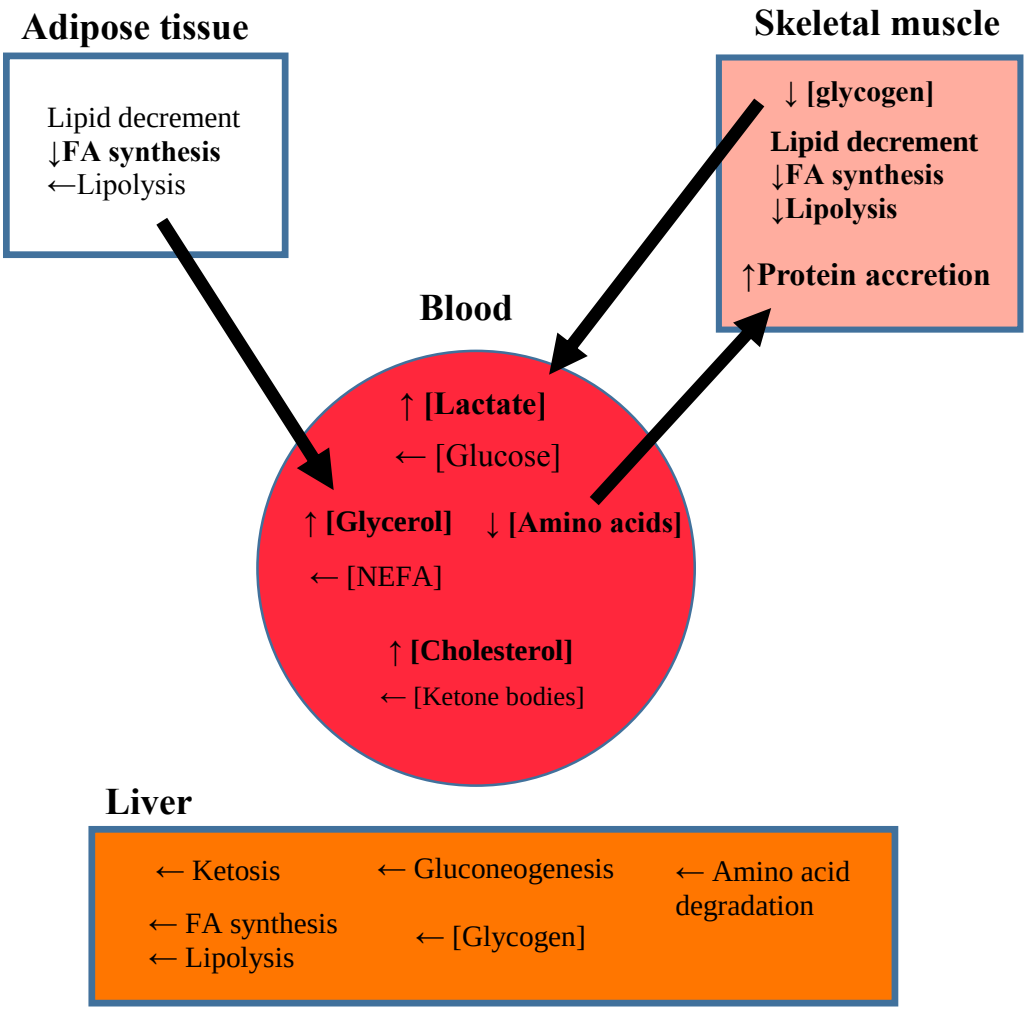


Figure 6.1: Schematic summary of the effects of the beta-agonist (BA) on sheep adipose tissue, skeletal muscle and liver as well as their relationship to circulating metabolites. Square brackets around text indicate a metabolite concentration arrows adjacent next to text indicate the changes in concentration or process; ↑ indicates an increase, ↓indicates a decrease and ← indicates no change relative to the control sheep. Solid arrows across tissue boundaries indicate possible absorption or secretion.

The reduction of FABP4 by dbcAMP treatment, also suggested the reduction of fat accumulation in the muscle, which might contribute the reduction of fat deposition, resulted in lean muscle production as demonstrated *in vivo* (de Almeida et al., 2012; Warriss et al., 1990; Fiems, 1987). Thus, taken together regarding the effects of BA *in vivo*, and dbcAMP *in vitro*, on myogenesis, and lipid metabolism, we suggested that both agents respond the similar pathway, *in vivo* or *in vitro*.

6.1.2 Effects of GH

Although there was no significant increase in muscle weights in the sheep treated with GH there were clear indications of effects on the liver. The increase in plasma IGF-1 suggested the effects of GH were, in part mediated by GH stimulated IGF-1. In the GH group, there was liver hypertrophy, which had been proposed to be caused by increased in glycogen and associated water content (Al-Doski, 2015). Our *in vitro* study involving the treatment of Des (1-3) IGF-1 on a differentiating myoblast cell line model, demonstrated the mRNA expression of myogenic-related genes was not affected, suggesting that the Des (1-3) IGF-1 was not strongly involved in regulating myogenesis, or hypertrophy effect as had been observed *in vivo*.

However, unlike BA, GH treatment of sheep clearly increased the mobilisation of NEFA into the plasma. This increase in NEFA plasma was associated with an apparent increase in ketogenesis as there was an associated increase in ketone bodies in the plasma. Such an increase is usually associated with a prolonged fasted state where NEFA are mobilised due to drop in insulin. However, in this study, there was elevated plasma glucose in the GH group indicating these animals potentially have a lack of insulin sensitivity. Apart of insulin sensitivity, this study also supported the Randle hypothesis, which postulated the competition between free fatty acids and glucose utilization in the muscle (Randle et al., 1963). As agreed with current study, the effects of increased plasma NEFAs from lipolysis, probably reduced the rate of glucose utilization in the muscles, which in turn accumulated in the plasma.

Metabolomics analysis demonstrated GH treatment generally reduced the circulating glucogenic amino acids concentration, which might be responsible for the increase in plasma glucose concentration. If these amino acids were being absorbed by the liver, they would be expected to promote gluconeogenesis. Therefore, it appeared in GH-treated lambs there was increased insulin resistance and an induced ‘diabetogenic’ effect. This was indicated by the increase in breakdown metabolite, beta-hydroxypyruvate, which demonstrated to associate with insulin resistance and diabetes (Mizuno, 2015; McDowell,

1983). Not all amino acids were affected by GH treatment, for example the plasma ketogenic amino acids, however GH induced the increase of plasma ketogenic amino acids breakdown metabolites, such as alpha-hydroxyisocaproate (HICA), which is the end product of leucine metabolism (Mero et al., 2010). The increase in circulating NEFAs is likely, due to suppression of fatty acids esterification and synthesis of lipids as well as reduction of antilipolytic effect of insulin (Saporano et al., 2015). There was reduction of lipogenic enzyme mRNA expression (decreased ACC and FAS gene expression) in subcutaneous fat as well as muscle in the current study has also been reported by others (Funai et al., 2013). This reduction in potential lipogenic capacity would also tend to lead to an accumulation of NEFAs. Although there no effect of GH on lipolytic mRNA in adipose tissue, the increase in circulation NEFAs and glycerol, might be due to lipolysis in visceral adipose tissue, rather than subcutaneous adipose tissue (Vijayakumar et al., 2011). In addition, many of these enzymes are activated by post-translational modification. The trend for GH to reduce lipolytic mRNA expression in muscles, as well as reducing lipogenic mRNA, is indicative of a reduction in lipid deposition, which would be beneficial in promoting lean muscle development. The effects of GH treatment *in vivo* according to the target organs were summarized in Figure 6.2.

Meanwhile, in our *in vitro* study investigating the effect of Des (1-3) IGF-1 treatment of differentiated C2C12 cells, the agent significantly increased PPARγ mRNA expression, confirming that IGF-1 treatment is involved in regulating adipogenesis. However, the agent did not have any effect on FABP4 mRNA expression, which is the markers for adipocytes, and the target genes for PPARγ, as well as CD36 mRNA expression, suggested differential pathway of adipogenesis, *in vivo* and *in vitro*, which could be mediated by direct GH effect or indirectly mediated by IGF-1. However, while GH strongly reduced FAS expression in muscles *in vivo*, reflecting reduced lipogenesis, the FAS expression *in vitro* was not significantly affected by Des (1-3) IGF-1, which probably reflects the lipogenic action of GH *in vivo* was mediated by direct GH effect, and might not mediated by IGF-1.

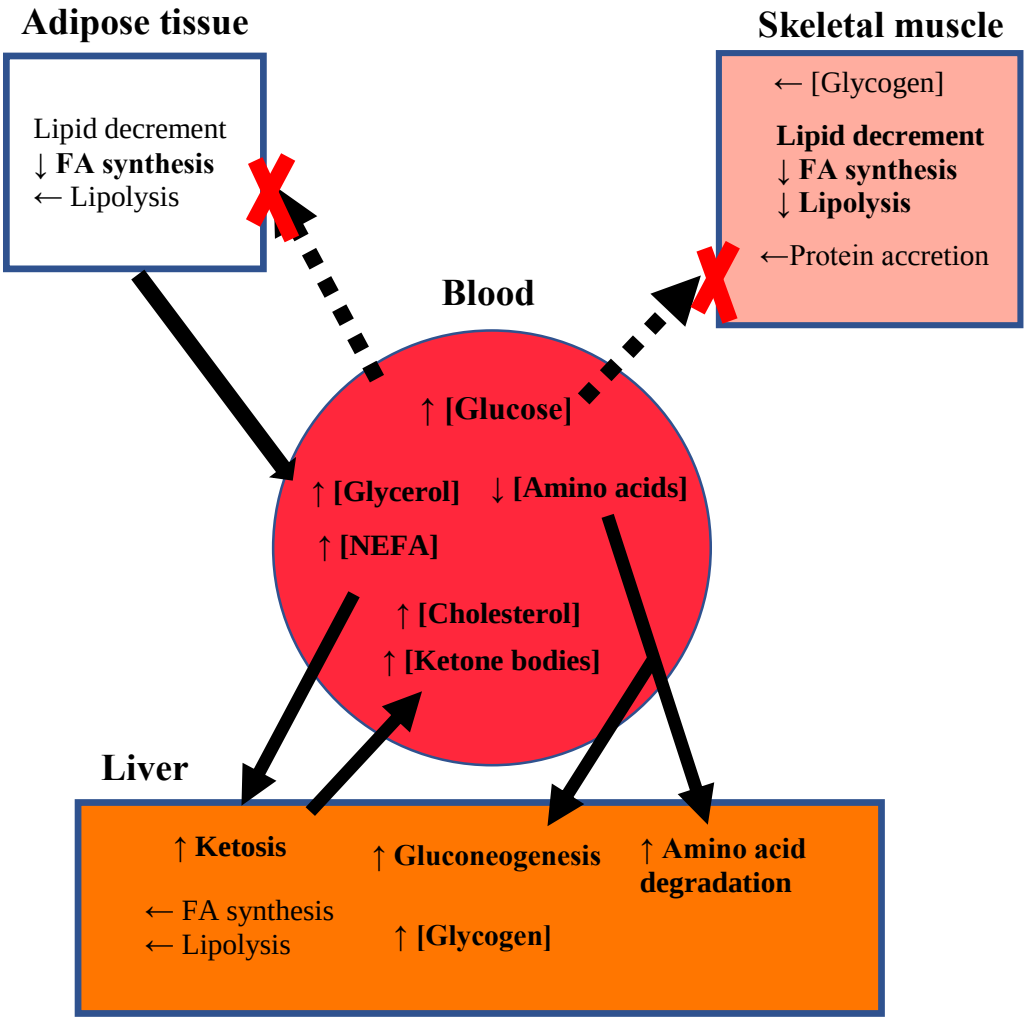


Figure 6.2: Schematic summary of the effects of the growth hormone (GH) on sheep adipose tissue, skeletal muscle and liver as well as their relationship to circulating metabolites. Square brackets around text indicate a metabolite concentration, arrows adjacent next to text indicate the changes in concentration or process; ↑ indicates an increase, ↓indicates a decrease and ← indicates no change relative to the control sheep. Dotted arrows indicate potential reduction in the absorption of glucose from the circulation. Solid arrows across tissue boundaries indicate possible absorption or secretion.

Taken together, we summarized that the effect of GH was more pronounced in lipid deposition and mobilization, while BA showed strong effect in muscle hypertrophy. However, both agents were exhibit similar response, particularly in reduced lipogenesis. In addition, dbcAMP and Des (1-3) IGF-1 were suggested to mediate the effects by BA and GH, respectively. The effects of dbcAMP and Des (1-3) IGF-1 *in vitro* on target mRNA expressions on were summarized in Figure 6.3.

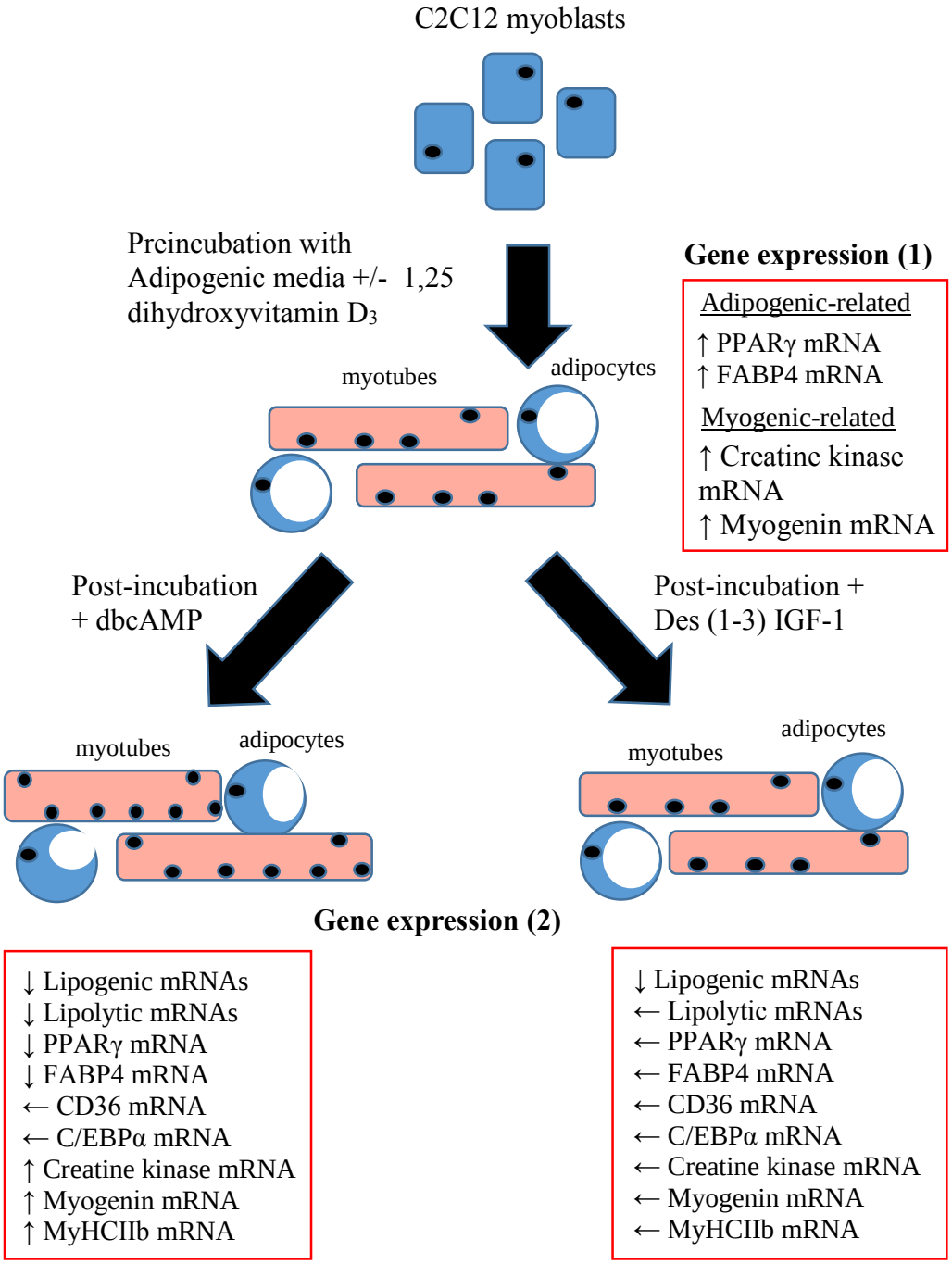


Figure 6.3: Schematic summary of the effects of the dbcAMP and Des (1-3) IGF-2 on the expression of adipogenic-related and myogenic-related genes in the transdifferentiated myoblast C2C12 cell lines. The arrows adjacent next to text indicate the changes in the mRNA expression; ↑ indicates an increase, ↓indicates a decrease and ← indicates no change. Solid arrows indicate the flow of the treatment consist of pre- and post-incubation processes. The gene expression in (1) indicates the mRNA expression during preincubation (with or without 1,25 dihydroxyvitamin D_3 in adipogenic media), while gene expression in (2) indicates mRNA expression during post-incubation (after treated with dbcAMP or Des (1-3) IGF-1).

6.2 GENERAL CONCLUSION

GH was demonstrated to reduce lipogenesis subcutaneous adipose tissues and muscles, while reducing the lipid turnover in the muscle. This appeared to induce the insulin resistance, which might contribute for the increase in lipid mobilization and increase lipid oxidation in GH treated animals. Meanwhile, the *in vitro* study demonstrated the novel development of intramuscular fat in myogenic cell lines. This model was suggested to be analogous to 'true' muscle, as seen *in vivo*, as it expressed both myogenic-related and adipogenic-related genes. On this model, dbcAMP reduced lipogenesis, while Des (1-3) IGF-1 appeared to induce adipogenesis. The role of BA in promoting myogenesis *in vivo*, was also demonstrated by the effect of dbcAMP *in vitro*, indicating by increased the expression of myogenic-related genes, and might reflect to the muscle hypertrophy. Generally, both agents appeared to reduce the lipid turnover in the muscles, which might contribute to the lean muscle development. This approach will have an impact in reducing harmful effects of fat in the muscles, which leads to increase healthy meat production, and beneficial for human health.

6.3 LIMITATIONS AND FUTURE WORK

Both of our *in vivo* and *in vitro* studies demonstrated the effects of GH in altering lipid deposition. However, certain genes were not measured in our *in vivo*, particularly in relating with adipogenesis, as similar with our *in vitro* study. The potential effects of those metabolic modifiers in altering the adipogenic gene expressions such as PPARγ, FABP4, CD36 and CEBPs could be beneficial in explaining the role of GH and BA in lipid metabolism. Meanwhile, the particular genes which involved in fatty acid development such as stearoyl CoA-desaturase (SCD) could determine the effects of both treatments in saturation or unsaturation fatty acid, which plays a big role in determining healthy meat.

While the intramuscular fat model on differentiated myogenic cell lines was established, further investigations on the metabolite concentration in the culture

media after treatment with the metabolic agents are required in order to ascertain the effects of the agents whether the lipolytic activity was induced. Such agents are glycerol and fatty acids, which are potential indicators of lipid turnover. In addition, determining media amino acids concentration and measure of the fibre formation could be indicate of the hypertrophic effect of the agents. As both agents potentially induced the mRNA expression either related with myogenesis or adipogenesis, the protein expression would be useful in order to interpret the exact effects of the agents, regarding posttranscriptional or posttranslational effects. Besides transdifferentiating, the cell culture model cold be developed by the incubation (co-culturing) of the muscle cells and fat cells in one culture model, and the effects of both agents could be determined by the same intervention. In addition, both agents could be used together (combined) as one treatment, and the effects could be compared with the effect of single agent, in the model. This could be beneficial on order to determine, whether the combined agents have a synergistic and strong effects in both myogenesis and adipogenesis, which could have impact in the production of good and healthy meat. This approach is also useful in order to determine the mechanism which might connect the cAMP/PKA (by the effect of BA/dbcAMP) and PI3K/AKt (by the effect of GH/IGF-1) pathways, which both are responsible in protein metabolism. The *in vitro* cell culture model described in this thesis could be used as a screening tool to examine the likely effects of metabolic agents on a mixed population of cells which more closely reflects *in vivo* muscle rather than a single cell type. The cell model would allow the segregation of factors which are often present together *in vivo* to determine their individual impact, for example the effects of GH or IGF-1. Importantly, such a cell model would allow mechanistic investigations of factors that influence muscle composition, particularly the impact on muscle fibres and fat, which is closely associated with meat quality.

7. APPENDICES

7.1 PRIMERS USED IN THE *IN VIVO* AND *IN VITRO* STUDIES

Table 7.1: Quantitative PCR primers for sheep transcripts

Target Gene	**Primer Sequence (5' to 3')**
ACTB	F: CTGGCACCACACCTTCTACAAC R: GTCTCAAACATGATCTGGGTCATC
ACC	F: GCCAACGCAGAATACATTAAGATG R: CAAGAATTAACTCCACATTTGCATAGTT
FAS	F: CCCAGCTCAACGAAACCA R: GACGAGGTCAACACCCTTCC
ATGL	F: GAGCTTATCCAGGCCAATGTCT R: CTCATAGAGCGGCAGGTTGTC
HSL	F: GGGGAGCACTACAAACGAAA R: CATAGGAGATGAGCCTGACGAG
ADIPO R2	F: GGCAACATCTGGACACATC R: CTGGAGACCCCTTCTGAG

F, forward; R, reverse, all primers are listed 5' to 3'

Table 7.2: Quantitative PCR primers for mouse transcripts

Target Gene	**Primer Sequence (5' to 3')**
ACC	F: CCATTGGTATTGGGGCTTAC R: CCGACCAAGGACTTTGTTG
FAS	F: CGGAAACTTCAGGAAATGTCC R: TCAGAGACGTGTCACTCCTGG
ATGL	F: CAACGCCACTCACATCTAC R GGACACCTCAATAATGTTG
HSL	F: GCGGCTGTCTAATGTCTTTGCA R: CCTTCATCACCCTCGAAGAAGA
PPARγ	F: GCATGGTGCCTTCGCTGA R: TGGCATCTCTGTGTCAACCATG
CD/36	F: AAACCCAGATGACGTGGC

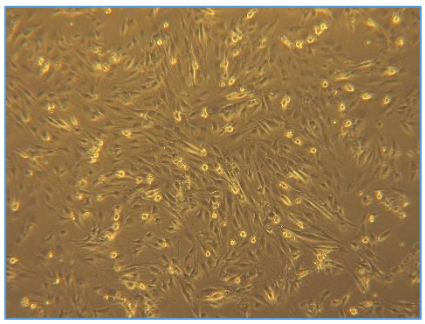
	R: AAGATGGCTCCATTGGGC
CEBPα	F: GCCAAACTGAGACTCTTC R: GGAAGCCTAAGTCTTAGC
FABP4	F: AAGTGGGAGTGGGCTTTGC R: TGGTGACCAAATCCCCATTT
Creatine kinase	F: GCACTGGCCGCAGCAT R: AGGGTAGTACTTGCCCTTGAACTC
Myf5	F: CAGCCCCACCTCCAACTG R: GCAGCACATGCATTTGATACATC
MyoD	F: CGTGGCAGCGAGCACTAC R: TGTAATCCATCATGCCATCAGA
Myogenin	F: CCCATGGTGCCCAGTGAA R: GCAGATTGTGGGCGTCTGTA
MyHCI	F: CTCAAGCTGCTCAGCAATCTATTT R: GGAGCGCAAGTTTGTCATAAGT
MyHCIIa	F: AGGCGGCTGAGGAGCACGTA R: GCGGCACAAGCAGCGTTGG
MyHCIIb	F: CAATCAGGAACCTTCGGAACAC R: GTCCTGGCCTCTGAGAGCAT
MyHCIIx	F: GAGGGACAGTTCATCGATAGCAA R: GGGCCAACTTGTCATCTCTCAT

F, forward; R, reverse, all primers are listed 5’ to 3’

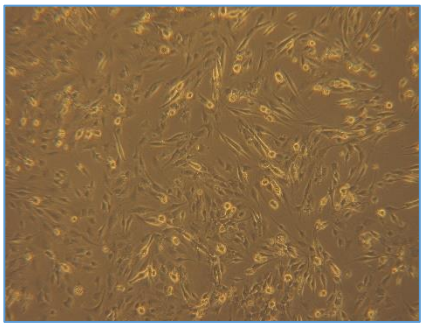
7.2 PHENOTYPE CHANGES OF C2C12 CELLS DURING 3- DAY TREATMENT WITH DBCAMP AND DES (1-3) IGF-1

Day 1

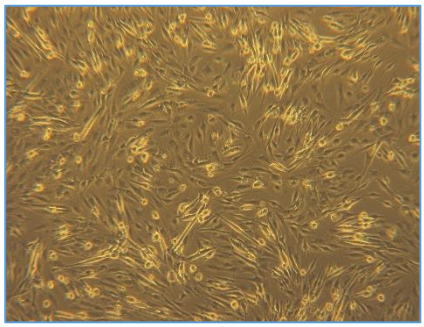
A) DM/Vit D-



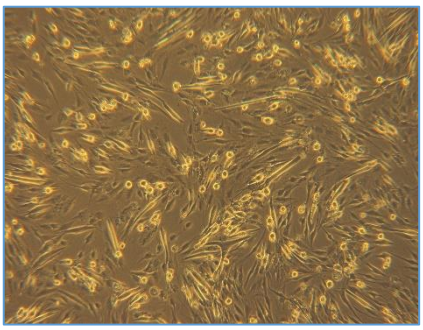
B) DM/Vit D+



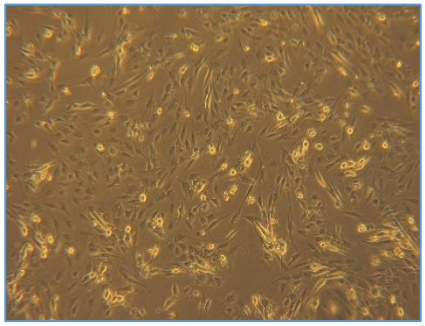
C) DB/Vit D-



D) DB/Vit D+



E) DES/Vit D-



F) DES/Vit D+

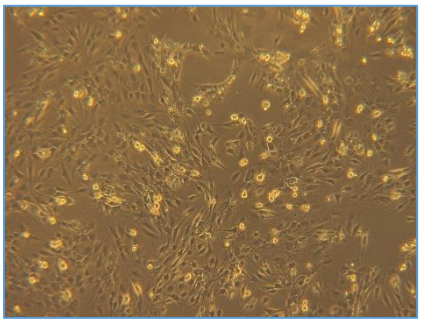
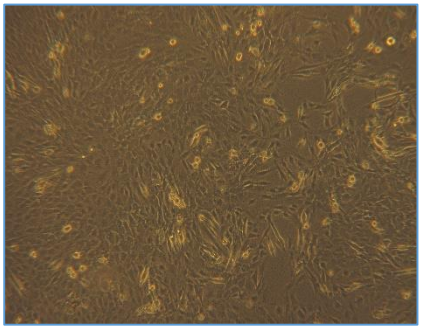


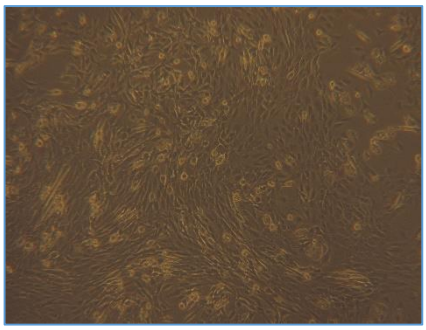
Figure 7.1: Effects of treatment either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on the phenotype changes of C2C12 cells on day 1 of treatment. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with vehicle control, DMSO or supplemented with dbcAMP or Des (1-3) IGF-1, with n=3 per treatment group.

Day 2

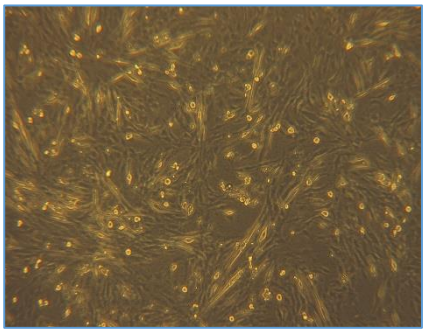
A) DM/Vit D-



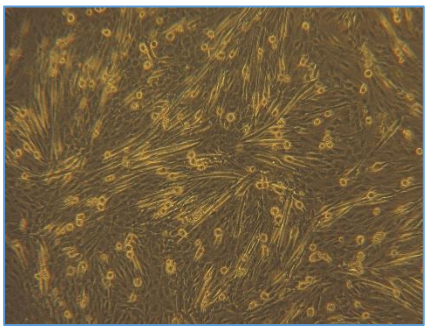
B) DM/Vit D+



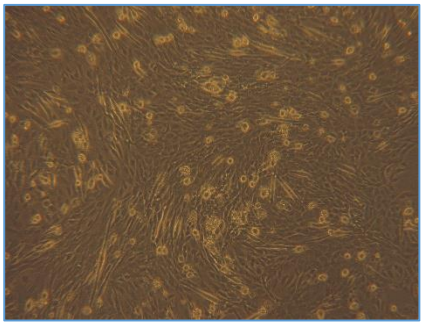
C) DB/Vit D-



D) DB/Vit D+



E) DES/Vit D-



F) DES/Vit D+

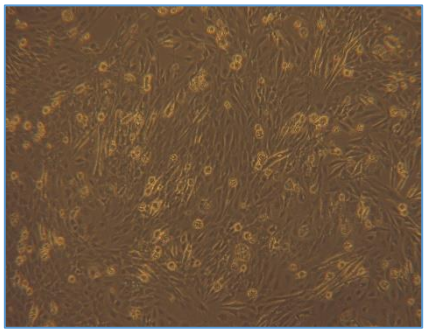
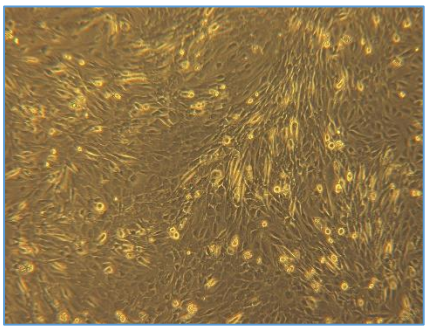


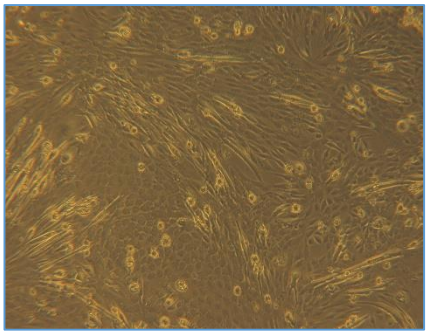
Figure 7.2: Effects of treatment either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on the phenotype changes of C2C12 cells on day 2 of treatment. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with vehicle control, DMSO or supplemented with dbcAMP or Des (1-3) IGF-1, with n=3 per treatment group.

Day 3

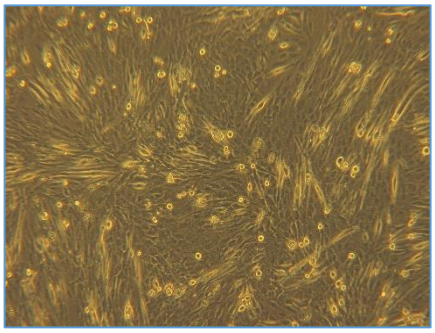
A) DM/Vit D-



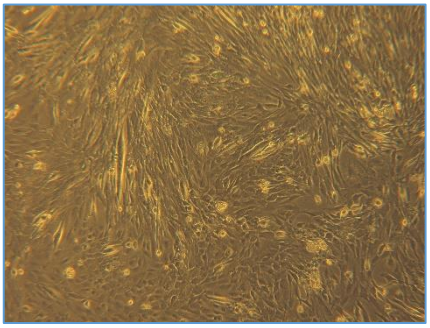
B) DM/Vit D+



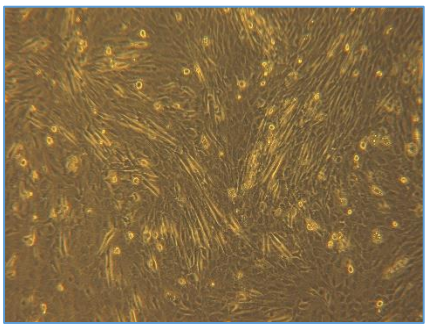
C) DB/Vit D-



D) DB/Vit D+



E) DES/Vit D-



F) DES/Vit D+

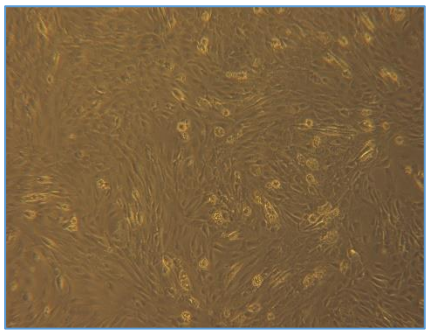


Figure 7.3: Effects of treatment either DMSO (DM), dbcAMP (DB) or Des (1-3) IGF-1 (DES), on the phenotype changes of C2C12 cells on day 3 of treatment. The mouse myogenic cell line, C2C12, were exposed to adipogenic media either containing DMSO, or 10^{-12} M $1,25(OH)_2D_3$ from the point of 70-80% confluence (day 0) for 6 days. Subsequently cells were then cultured for 1, 2 or 3 days in adipogenic media with 10^{-12} M of $1,25(OH)_2D_3$ (Vit D+) or without 10^{-12} M $1,25(OH)_2D_3$ (Vit D-) with vehicle control, DMSO or supplemented with dbcAMP or Des (1-3) IGF-1, with n=3 per treatment group.

8 REFERENCES

1. AbouHaidar, M.G., and Ivanov, I.G. (1999). Non-enzymatic RNA hydrolysis promoted by the combined catalytic activity of buffers and magnesium ions. *Journal of Biosciences*. **54**, 542-548.
2. Accili, D., Nakae, J., Kim, J.J., Park, B.C., and Rother, K.I. (1999). Targeted gene mutations define the roles of insulin and IGF-I receptors in mouse embryonic development. *Journal of Paediatric Endocrinology and Metabolism* **12**, 475-485.
3. Ahrén, B., Järhult, J., and Lundquist, I. (1981). Insulin secretion induced by glucose and by stimulation of beta 2 -adrenoceptors in the rat. Different sensitivity to somatostatin. *Acta Physiologica Scandinavica* **112**, 421-426.
4. Al-Doski, S. (2015). *Effects of growth promoters on sheep metabolism and growth*. PhD thesis, University of Nottingham.
5. Ali, A.T., Hochfeld, W.E., Myburgh, R., and Pepper, M.S. (2013). Adipocyte and adipogenesis. *European Journal of Cell Biology* **92**, 229-236.
6. Ameer, F., Scandiuzzi, L., Hasnain, S., Kalbacher, H., and Zaidi, N. (2014). *De novo* lipogenesis in health and disease. *Metabolism* **63**, 895-902.
7. André, C., Couton, D., Gaston, J., Erraji, L., Renia, L., Varlet, P., Briand, P., and Guillet, J.G. (1999). β_2-adrenergic receptor-selective agonist clenbuterol prevents Fas-induced liver apoptosis and death in mice. *The American Journal of Physiology* **276,** G647-G654.
8. Arimura, N., Horiba, T., Imagawa, M., Shimizu, M., and Sato, R. (2004). The peroxisome proliferator-activated receptor regulates expression of the perilipin gene in adipocytes. *The Journal of Biological Chemistry* **279**, 10070–10076.
9. Awede, B.L., Thissen, J.P., and Lebacq, J. (2002). Role of IGF-I and IGFBPs in the changes of mass and phenotype induced in rat soleus muscle by clenbuterol. *The American Journal of Physiology - Endocrinology and Metabolism* **282**, E31-E37.
10. Ballard, F.J., Wallace, J.C., Francis, G.L., Read, L.C., and Tomas, F.M. (1996). Des (1-3) IGF-I: A truncated form of insulin-like growth factor-I. *The International Journal of Biochemistry and Cell Biology* **28**, 1085-1087.
11. Barisione, G., Baroffio, M., Crimi, E., and Brusasco, V. (2010). Beta-adrenergic agonists. *Pharmaceuticals* **3**, 1016–1044.

12. Barshevskaia, T.N., Goriunova, L.E., and Bibilashvili, R.S. (1987). Non-specific RNA degradation in the presence of divalent magnesium ions. *Molecular Biology* **21**, 1235-1241.
13. Bates, P.C., and Pell, J.M. (1991). Action and interaction of growth hormone and the β-agonist, clenbuterol, on growth, body composition and protein turnover in dwarf mice. *British Journal of Nutrition* **65**, 115-129.
14. Baumann, G., Amburn, K., and Shaw, M.A. (1988). The circulating growth hormone binding protein complex: A major constituent of plasma growth hormone in man. *Endocrinology* **122**, 976–984.
15. Beederman, M., Lamplot, J. D., Nan, G., Wang, J., Liu, X., Yin, L., Li, R., Shui, W., Zhang, H., Kim, S.H., Zhang, W., Zhang, J., Kong, Y., Denduluri, S., Rogers, M.R., Pratt, A., Haydon, R.C., Luu, H.H., Angeles, J., Shi, L.L., and He, T-C. (2013). BMP signaling in mesenchymal stem cell differentiation and bone formation. *Journal of Biomedical Science and Engineering* **6**, 32–52.
16. Beermann, D.H., Butler, W.R., Hogue, D.E., Fishell, V.K., Dalrymple, R.H., Ricks, C.A., and Scanes, C.G. (1987). Cimaterol-induced muscle hypertrophy and altered endocrine status in lambs. *Journal of Animal Science* **65**, 1514-1524.
17. Beermann, D.H., Campion, D.R., and Dalrymple, R.H. (1985). Mechanisms responsible for partitioning tissue growth in meat animals. *38th Reciprocal Meat Conference Proceedings* **38**, 105-114.
18. Bender, A. (1992). Meat and meat products in human nutrition in developing countries. FAO Food and Nutrition Paper no. 53. Rome: FAO.
19. Bentzinger, C.F., Wang, Y.X., and Rudnicki, M.A. (2012). Building muscle: molecular regulation of myogenesis. *Cold Spring Harbor Perspectives in Biology* **4**.
20. Benvenuti, S., Cellai, I., Luciani, P., Deledda, C., Baglioni, S., Giuliani, C., Saccardi, R., Mazzanti, B., Dal Pozzo, S., Mannucci, E., Peri, A., and Serio, M. (2007). Rosiglitazone stimulates adipogenesis and decreases osteoblastogenesis in human mesenchymal stem cells. *Journal of Endocrinological Investigation* **30**, RC26-RC30.
21. Bernard, P.S., and Wittwer, C.T. (2002). Real-time PCR technology for cancer diagnostics. *Clinical Chemistry* **48**, 1178-1185.

22. Beswick, N.S., and Kennelly, J.J. (1998). The influence of bovine growth hormone and growth hormone releasing factor on acetyl-CoA carboxylase and fatty acid synthase in primiparous Holstein cows. *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology and Endocrinology* **120**, 241–249.
23. Bezaire, V., Mairal, A., Ribet, C., Lefort, C., Girousse, A., Jocken, J., Laurencikiene, J., Anesia, R., Rodriguez, A.M., Ryden, M., Stenson, B.M., Dani, C., Ailhaud, G., Arner, P., and Langin, D. (2009). Contribution of adipose triglyceride lipase and hormone-sensitive lipase to lipolysis in hMADS adipocytes. *The Journal of Biological Chemistry* **284**, 18282-18291.
24. Bi, X, and Henry, C.J. (2017). Plasma-free amino acid profiles are predictors of cancer and diabetes development. *Nutrition and Diabetes* **7**, e249.
25. Blanchard, P.G., Festuccia, W.T., Houde, V.P., St-Pierre, P., Brûlé, S., Turcotte, V., Côté, M., Bellmann, K., Marette, A., and Deshaies, Y. (2012). Major involvement of mTOR in the PPARγ-induced stimulation of adipose tissue lipid uptake and fat accretion. *Journal of Lipid Research* **53**, 1117-1125.
26. Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911-917.
27. Blum, J.W., and Flueckiger N. (1988). Early metabolic and endocrine effects of perorally administered beta-adrenoceptor agonists in calves. *European Journal of Pharmacology* **151**, 177-187.
28. Blum, J.W., Froehli, D., and Kunz, P. (1982). Effects of catecholamines on plasma free fatty acids in fed and fasted cattle. *Endocrinology* **110**, 452-456.
29. Borycki, A. G., Brunk, B., Tajbakhsh, S., Buckingham, M., Chiang, C., and Emerson, C.P., Jr. (1999) Sonic hedgehog controls epaxial muscle determination through Myf5 activation. *Development* **126**, 4053–4063.
30. Borycki, A-G., Brown, A.M.C., and Emerson, C.P.J. (2000). Shh and Wnt signaling pathways converge to control Gli gene activation in avian somites. *Development* **127**, 2075-2087.
31. Brameld, J.M., Atkinson, J.L., Saunders, J.C., Pell, J.M., Buttery, P.J., and Gilmour, R.S. (1996). Effects of growth hormone administration and dietary protein intake on insulin-like growth factor I and growth hormone receptor

mRNA expression in porcine liver, skeletal muscle, and adipose tissue. *Journal of Animal Science* **74**, 1832-1841.

32. Braun, T., and Arnold, H.H. (1995). Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. *EMBO Journal* **14**, 1176–1186.
33. Braun, T., Rudnicki, M.A., Arnold, H.H., and Jaenisch, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell* **71**, 369-382.
34. Brown, D.M., Parr, T., and Brameld, J.M. (2011). Myosin heavy chain mRNA isoforms are expressed in two distinct cohorts during C2C12 myogenesis. *Journal of Muscle Research and Cell Motility* **32**, 383-390.
35. Brownsey, R.W., Boone, A.N., Elliott, J.E., Kulpa, J.E., and Lee, W.M. (2006). Regulation of acetyl-CoA carboxylase. *Biochemical Society Transactions* **34**, 223-227.
36. Brun, R.P., Tontonoz, P., Forman, B.M., Ellis, R., Chen, J., Evans, R.M., and Spiegelman, B.M. (1996). Differential activation of adipogenesis by multiple PPAR isoforms. *Genes and Development* **10**, 974-984.
37. Buckway, C.K., Guevara-Aguirre, J., Pratt, K.L., Burren, C.P., and Rosenfeld, R.G. (2001). The IGF-I generation test revisited: A marker of GH sensitivity. *The Journal of Clinical Endocrinology and Metabolism* **86**, 5176-5183.
38. Burattini, S., Ferri, P., Battistelli, M., Curci, R., Luchetti, F., and Falcieri, E. (2004). C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. *European Journal of Histochemistry* **48**, 223-233.
39. Bustin, S.A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology* **29**, 23-39.
40. Cao, Z., Umek, R.M., and McKnight, S.L. (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes and Development* **5**, 1538 –1552.
41. CAST. (2005). Metabolic Modifiers for Use in Animal Production. *Animal Agriculture's Future through Biotechnology* **30**.
42. Ceglia, L., and Harris, S.S. (2013). Vitamin D and its role in skeletal muscle. *Calcified Tissue International* **92**, 151-162.

43. Cersosimo, E., Danou, F., Persson, M., and Miles, J.M. (1996). Effects of pulsatile delivery of basal growth hormone on lipolysis in humans. *American Journal of Physiology* **271**, E123-E126.
44. Chamberlain, J.S., Farwell, N.J., Chamberlain, J.R., Cox, G.A., and Caskey, C.T. (1991). PCR analysis of dystrophin gene mutation and expression. *Journal of Cellular Biochemistry* **46**, 255-259.
45. Chang, E., and Kim, Y. (2016). Vitamin D decreases adipocyte lipid storage and increases NAD-SIRT1 pathway in 3T3-L1 adipocytes. *Nutrition* **2**, 702-708.
46. Chang, J.H., Lin, H.K., Chung, H.S., Chang, Y.J., Chi, H.C., and Chen, S.L. (2006). Myogenic bHLH proteins regulate the expression of PPARc coactivator-1a. *Endocrinology* **147**, 3093–3106.
47. Chaves, V.E., Júnior, F.M., and Bertolini, G.L. (2013). The metabolic effects of growth hormone in adipose tissue. *Endocrine* **44**, 293-302.
48. Chirala, S.S., Jayakumar, A., Gu, Z-W., and Wakil, S.J. (2001). Human fatty acid synthase: Role of interdomain in the formation of catalytically active synthase dimer. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 3104–3108.
49. Chmurzyńska, A. (2006). The multigene family of fatty acid-binding proteins (FABPs): function, structure and polymorphism. *Journal of Applied Genetics*. **47**, 39–48.
50. Choi, Y.M. and Kim, B.C. (2009). Muscle fibre characteristics, myofibrillar protein isoforms, and meat quality. *Livestock Science* **122**, 105–118.
51. Choo, J.J., Horan, M.A., Little, R.A., and Rothwell, N.J. (1992). Anabolic effects of clenbuterol on skeletal muscle are mediated by beta 2-adrenoceptor activation. The *American Journal of Physiology* **263**, E50-E56.
52. Christiaens, V., Van Hul, M., Lijnen, H.R., and Scroyen, I. (2012). CD36 promotes adipocyte differentiation and adipogenesis. *Biochimica et Biophysica Acta* **1820**, 949-956.
53. Chruscinski, A.J., Rohrer, D.K., Schauble, E., Desai, K.H., Bernstein, D., and Kobilka, B.K. (1999). Targeted disruption of the β_2-adrenergic receptor gene. *The Journal of Biological Chemistry* **274**, 16694-16700.
54. Chung, C.S., Etherton, T.D., and Wiggins, J.P. (1985). Stimulation of swine growth by porcine growth hormone. *Journal of Animal Science* **60**, 118-130.

55. Chwalibog, A., Jensen, K., and Thorbek, G. (1996). Oxidation of nutrients in bull calves treated with β-adrenergic agonists. *Archiv für Tierernaehrung* **49,** 255-261.
56. Cisternas, P., Henriquez, J.P., Brandan, E., and Inestrosa, N.C. (2014). Wnt signaling in skeletal muscle dynamics: myogenesis, neuromuscular synapse and fibrosis. *Molecular Neurobiology* **49**, 574-589.
57. Claeys, M.C., Mulvaney, D.R., McCarthy, F.D., Gore, M.T., Marple, D.N., and Sartin, J.L. (1989). Skeletal muscle protein synthesis and growth hormone secretion in young lambs treated with clenbuterol. *Journal of Animal Science* **67**, 2245-54.
58. Clarke, S.D. (1993). Regulation of fatty acid synthase gene expression: An approach for reducing fat accumulation. *Journal of Animal Science* **71**, 1957-1965.
59. Clarkson, R.W., Chen, C.M., Harrison, S., Wells, C., Muscat, G.E., Waters, M.J. (1995). Early responses of trans-activating factors to growth hormone in preadipocytes: Differential regulation of CCAAT enhancer-binding protein-beta (C/EBP beta) and C/EBP delta. *Molecular Endocrinology* **9**, 108–120.
60. Claycombe, K.J., Jones, B.H., Standridge, M.K., Guo, Y., Chun, J.T., Taylor, J.W., and Moustaïd-Moussa, N. (1998). Insulin increases fatty acid synthase gene transcription in human adipocytes. *The American Journal of Physiology* **274**, R1253-R1259.
61. Coe, N.R., and Bernlohr, D.A. (1998). Physiological properties and functions of intracellular fatty acid-binding proteins. *Biochimica et Biophysica Acta* **1391**, 287-306.
62. Colbert, C.L., Kim, C.W., Moon, Y.A., Henry, L., Palnitkar, M., McKean, W.B., Fitzgerald, K., Deisenhofer, J., Horton, J.D., and Kwon, H.J. (2010). Crystal structure of Spot 14, a modulator of fatty acid synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 18820-18825.
63. Cossu, G., and Borello, U. (1999). Wnt signaling and the activation of myogenesis in mammals. *EMBO Journal* **18**, 6867-6872.
64. Crespo, P., Cachero, T.G., Xu, N. and Gutkind, J.S. (1995). Dual effect of β-adrenergic receptors on mitogen activated protein kinase: Evidence for a bγ-

dependent activation and Ga_s-cAMP-mediated inhibition. *The Journal of Biological Chemistry* **270**, 25259–25265.

65. Daaka, Y., Luttrell, L.M., and Lefkowitz, R.J. (1997). Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390**, 88-91.
66. Dalen, K.T., Schoonjans, K., Ulven, S.M., Weedon-Fekjaer, M.S., Bentzen, T.G., Koutnikova, H., Auwerx, J., and Nebb, H.I. (2004). Adipose tissue expression of the lipid droplet-associating proteins S3-12 and perilipin is controlled by peroxisome proliferator-activated receptor-γ. *Diabetes* **53**, 1243-1252.
67. de Almeida, V.V., Nuñez, A.J.C., and Miyada, V.S. (2012). Ractopamine as a metabolic modifier feed additive for finishing pigs: A review. *Brazilian Archives of Biology and Technology* **55**, 445-456.
68. de Brun, V., Meikle, A., Casal, A., Sequeira, M., Contreras-Solís, I., Carriquiry, M., Forcada, F., Sosa, C., and Abecia, J.A. (2015). Periconceptional undernutrition modifies endocrine profiles and hepatic gene expression in sheep. *Journal of Animal Physiology and Animal Nutrition* **99**, 710-718.
69. del Barrio, A.S., García-Calonge, M.A., Fernández-Quintela, A., Simón, E., Portillo, M.P., Astiasarán, I., and Martínez, J.A. (1995). Effects of the beta-adrenergic agonist salbutamol and its withdrawal on protein metabolism of lambs. *Annals of Nutrition and Metabolism* 39, 317-324.
70. Del Boca, J., and Flatt, J.P. (1969). Fatty acid synthesis from glucose and acetate and the control of lipogenesis in adipose tissue. *European Journal of Biochemistry* **11**, 127-134.
71. Delgado, C.L. (2003). Rising consumption of meat and milk in developing countries has created a new world food revolution. *The Journal of Nutrition* **133**, 3907S–3910S.
72. Dietz, J., and Schwartz, J. (1991). Growth hormone alters lipolysis and hormone-sensitive lipase activity in 3T3-F442A adipocytes. *Metabolism* **40**, 800-806.
73. Dikeman, M., and Devine, C. (2014). *Encyclopedia of Meat Sciences: 2nd Edition.* Retrieved from http://books.google.co.uk
74. Dikeman, M.E. (2003). Metabolic modifiers and genetics: Effects on carcass traits and meat quality. *Brazilian Journal of Food Technology* **6**, 1–38.

75. Dikeman, M.E. (2007). Effects of metabolic modifiers on carcass traits and meat quality. *Meat Science* **77**, 121–135.
76. Doglio, A., Dani, C., Grimaldi, P., and Ailhaud, G. (1986). Growth hormone regulation of the expression of differentiation-dependent genes in preadipocyte Ob1771 cells. *Biochemical Journal* **238**, 123–129.
77. Doglio, A., Dani, C., Grimaldi, P., and Ailhaud, G. (1989). Growth hormone stimulates c-fos gene expression by means of protein kinase C without increasing inositol lipid turnover. *Proceedings of the National Academy of Sciences of the United State of America* **86**, 1148–1152.
78. Dominici, F.P., Cifone, D., Bartke, A., and Turyn, D. (1999). Alterations in the early steps of the insulin-signaling system in skeletal muscle of GH-transgenic mice. *The American Journal of Physiology* **277**, E447-E454.
79. Donkin, S.S., Chiu, P.Y., Yin, D., Louveau, I., Swencki, B., Vockroth, J., Evock-Clover, C.M., Peters, J.L., and Etherton, T.D. (1996). Porcine somatotropin differentially down-regulates expression of the GLUT4 and fatty acid synthase genes in pig adipose tissue. *The Journal of Nutrition*. **126**, 2568-2577.
80. dos Santos, C.M.A., van Kerkhof, P., and Strous, G.J. (2001). The signal transduction of the growth hormone receptor is regulated by the ubiquitin/proteasome system and continues after endocytosis. *The Journal of Biological Chemistry* **276**, 10839-10846.
81. Dotti, I. and Bonin, S. (2011). *DNase treatment of RNA. Guidelines for molecular analysis in archive tissues.* G. Stanta. Berlin, Heidelberg, Springer Berlin Heidelberg: 87-90.
82. Du, M., Wang, B., Fu, X., Yang, Q., and Zhu, M.J. (2015). Fetal programming in meat production. *Meat Science* **109**, 40–47.
83. Duncan, R.E., Ahmadian, M., Jaworski, K., Sarkadi-Nagy, E., and Sul, H.S. (2007). Regulation of lipolysis in adipocytes. *Annual Review of Nutrition* **27**, 79–101.
84. Dunshea, F.R., and King, R.H. (1995). Responses to homeostatic signals in ractopamine-treated pigs. *British Journal of Nutrition* **73**, 809-818.
85. Dunshea, F.R., D'Souza, D.N., Pethick, D.W., Harper, G.S., and Warner, R.D. (2005). Effects of dietary factors and other metabolic modifiers on quality and nutritional value of meat. *Meat Science* **71**, 8–38.

86. Dunshea, F.R., D'Souza, D.N., and Channon, H.A. (2016). Metabolic modifiers as performance-enhancing technologies for livestock production. *Animal Frontiers* **6**, 6–14.
87. Duquette, P.F., and Muir, L.A. (1984). Effects of ovine growth hormone and other anterior pituitary hormones on lipolysis of rat and ovine adipose tissue *in vitro*. *Journal of Animal Science*. **58**, 1191-1197.
88. Duquette, P.F., and Muir, L.A. (1985). Effect of the β-adrenergic agonists isoproterenol, clenbuterol, L-640,033 and BRL35135 on lipolysis and lipogenesis in rat adipose tissue *in vitro*. *Journal of Animal Science* **61**, 265.
89. Eadara, J.K., Dalrymple, R.H., DeLay, R.L., Ricks, C.A., and Romsos, D.R. (1989). Effects of cimaterol, a β-adrenergic agonist, on lipid metabolism in rats. *Metabolism* **38**, 522-529.
90. Eguizabal, C., Montserrat, N., Veiga, A., and Izpisua Belmonte, J.C. (2013). Dedifferentiation, transdifferentiation, and reprogramming: future directions in regenerative medicine. *Seminars in Reproductive Medicine* **31**, 82-94.
91. Eisemann, J.H., Huntington, G.B., and Ferrell, C.L. (1988). Effects of dietary clenbuterol on metabolism of the hindquarters in steers. *Journal of Animal Science* **66**, 342-353.
92. Elmasri, H., Karaaslan, C., Teper, Y., Ghelfi, E., Weng, M., Ince, T.A., Kozakewich, H., Bischoff, J., and Cataltepe S. (2009). Fatty acid binding protein 4 is a target of VEGF and a regulator of cell proliferation in endothelial cells. *FASEB Journal* **23**, 3865–3873.
93. Emery, P.W., Rothwell, N.J., Stock, M.J., and Winter, P.D. (1984). Chronic effects of β_2-adrenergic agonists on body composition and protein synthesis in the rat. *Bioscience Reports* **4**, 83-91.
94. Endo, T. (2015). Molecular mechanisms of skeletal muscle development, regeneration, and osteogenic conversion. *Bone* **80**, 2-13.
95. Eng, D., Ma, H-Y., Gross, M.K., and Kioussi, C. (2013). Gene networks during skeletal myogenesis. *ISRN Developmental Biology* **2013,** 1-8.
96. Ensinger H., Lindner, K.H., Dirks, B, Kilian, J., Gruenert, A., and Ahnefeld, F.W. (1992). Adrenaline: relationship between infusion rate, plasma concentration, metabolic and hemodynamic effects in volunteers. *European Journal of Anaesthesiology* **9**, 435–446.

97. Erbay, E., and Chen, J. (2001). The mammalian target of rapamycin regulates C2C12 myogenesis via a kinase-independent mechanism. *The Journal of Biological Chemistry* **276**, 36079-36082.
98. Erraji-Benchekroun, L., Couton, D., Postic, C., Borde, I., Gaston, J., Guillet, J.G., and André C. (2005). Overexpression of β_2-adrenergic receptors in mouse liver alters the expression of gluconeogenic and glycolytic enzymes. *America Journal of Physiology. Endocrinology and Metabolism* **288**, E715-E722.
99. Etherton, T.D., and Bauman, D.E. (1998). Biology of somatotropin in growth and lactation of domestic animals. *Physiological Reviews* **78**, 745-761.
100. Etherton, T.D., Louveau, I., Sørensen, M.T., and Chaudhuri, S. (1993). Mechanisms by which somatotropin decreases adipose tissue growth. *American Journal of Clinical Nutrition* **58**, 287S-295S.
101. FAO. (2009a). How to Feed the World in 2050. Rome.
102. FAO. (2013). Food systems for better nutrition. Rome.
103. Farmer, S.R. (2005). Regulation of PPARγ activity during adipogenesis. *International Journal of Obesity and Related Metabolic Disorders* **29**, S13-S16.
104. Farmer, S.R. (2006). Transcriptional control of adipocyte formation. *Cell Metabolism* **4**, 263–273.
105. Fennessy, P.F., McEwan, J.C., Lord, E.A., Greer, G.J., Bain, W.E., Johnstone, P.D., Knowler, M.A., Dalrymple, R.H., and Ingle, D.L. (1990). Effect of Cimaterol implants on lamb growth and carcass traits. *New Zealand Journal of Agricultural Research* **33**, 413-427.
106. Ferreira, M.S.S., Garbossa, C.A.P., Oberlender, G., Pereira, L.J., Zangeronimo, M.G., Sousa R.V., and Cantarelli, V.S. (2013). Effect of ractopamine on lipid metabolism in vivo – A systematic review. *Brazilian Archives of Biology and Technology* **56**, 35–43.
107. Ferrier, D.R. (2014). *Lippincott's Illustrated Reviews: Biochemistry*. Sixth Edition. Philadelphia, PA. Lippincott Williams and Wilkins.
108. Fève, B., Emorine, L.J., Lasnier, F., Blin, N., Baude, B., Nahmias, C., Strosberg, A.D., and Pairault, J. (1991). Atypical β-adrenergic receptor in 3T3-F442A adipocytes. *The Journal of Biological Chemistry* **266**, 20329−20336.

109. Fiems, L.O. (1987). Effect of beta-adrenergic agonists in animal production and their mode of action. *Annales de Zootechnie, INRA/EDP Sciences* **36**, 271-290.
110. Flint, D.J., Binart, N., Kopchick, J., and Kelly P. (2003). Effects of growth hormone and prolactin on adipose tissue development and function. *Pituitary* **6**, 97-102.
111. Folch, J., Ascoli, I., Lees, M., Meath, J.A., and LeBaron, F.N. (1951). Preparation of lipid extracts from brain tissue. *The Journal of Biological Chemistry* **191**, 833–841.
112. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry* **226**, 497–509.
113. Frayn, K.N., Arner, P., and Yki-Järvinen, H. (2006). Fatty acid metabolism in adipose tissue, muscle and liver in health and disease. *Essays in Biochemistry* **42**, 89-103.
114. Fredrikson, G., Tornqvist, H., and Belfrage, P. (1986). Hormone-sensitive lipase and monoacylglycerol lipase are both required for complete degradation of adipocyte triacylglycerol. *Biochimica et Biophysica Acta* **876**, 288-293.
115. Frontera, W., and Ochala, J. (2014). Skeletal Muscle: A brief review of structure and function. *Calcified Tissue International* **96**.
116. Fu, Y., Luo N., and Lopes-Virella M.F. (2000). Oxidized LDL induces the expression of ALBP/aP2 mRNA and protein in human THP-1 macrophages. *Journal of Lipid Research* **41**, 2017–2023.
117. Funai, K., Song, H., Yin, L., Lodhi, I.J., Wei, X., Yoshino, J., Coleman, T., and Semenkovich, C.F. (2013). Muscle lipogenesis balances insulin sensitivity and strength through calcium signaling. *The Journal of Clinical Investigation* **123**, 1229–1240.
118. Furuhashi, M., Saitoh, S., Shimamoto, K., and Miura, T. (2014). Fatty Acid-Binding Protein 4 (FABP4): Pathophysiological insights and potent clinical biomarker of metabolic and cardiovascular diseases. *Clinical Medicine Insights.* Cardiology **8**, 23–33.
119. Gall, W.E., Beebe, K., Lawton, K.A., Adam, K.P., Mitchell, M.W., Nakhle, P.J., Ryals, J.A., Milburn, M.V., Nannipieri, M., Camastra, S., Natali, A., Ferrannini, E.; RISC Study Group. (2010). α-hydroxybutyrate is an early

biomarker of insulin resistance and glucose intolerance in a nondiabetic population. *PLoS ONE* **5**, e10883.

120. Gan, L., Liu, Z., Cao, W., Zhang, Z., and Sun, C. (2015). FABP4 reversed the regulation of leptin on mitochondrial fatty acid oxidation in mice adipocytes. *Scientific Reports* **5**, 13588.
121. Garbossa, C.A.P., de Sousa, R.V., Cantarelli, V.D.S., Pimenta, M.E.D.S.G, Zangeronimo, M.G., Silveira, H., Kuribayashi, T.H., and Cerqueira, L.G.D.S. (2013). Ractopamine levels on performance, carcass characteristics and quality of pig meat. *Revista Brasileira de Zootecnia* **42**, 325-333.
122. Gebre, B.A., Gebretsadik, Z.T., and Anal, A.K. (2012) Effect of metabolic modifiers on meat quantity and quality. *African Journal of Food Science* **6**, 294-301.
123. Girgis, C.M., Clifton-Bligh, R.J., Mokbel, N., Cheng, K., and Gunton, J.E. (2014). Vitamin D signaling regulates proliferation, differentiation, and myotube size in C2C12 skeletal muscle cells. *Endocrinology* **155**, 347-357.
124. Glass, D.J. (2003). Molecular mechanisms modulating muscle mass. *Trends in Molecular Medicine* **9**, 344-350.
125. Glass, D.J. (2005). Skeletal muscle hypertrophy and atrophy signalling pathways. *The International Journal of Biochemistry and Cell Biology* **37**, 1974-1984.
126. Goldberg, I.J., Eckel, R.H., and Abumrad, N.A. (2009). Regulation of fatty acid uptake into tissues: lipoprotein lipase- and CD36-mediated pathways. *Journal of Lipid Research* **50**, S86-S90.
127. Gondret, F., Damon, M., Jadhao, S.B., Houdebine, L.M., Herpin, P., and Hocquette, J.F. (2004b). Age-related changes in glucose utilization and fatty acid oxidation in a muscle-specific manner during rabbit growth. *Journal of Muscle Research and Cell Motility* **25**, 405–410.
128. Gondret, F., Hocquette, J.F., and Herpin, P. (2004a). Age-related relationships between muscle fat content and metabolic traits in growing rabbits. *Reproduction Nutrition and Development* **44**, 1–16.
129. Goodman, H.M. (1963). Effects of chronic growth hormone treatment on lipogenesis by rat adipose tissue. *Endocrinology* **72**, 95-99.

130. Gopinath, R., and Etherton, T.D. (1989). Effects of porcine growth hormone on glucose metabolism of pigs: I. Acute and chronic effects on plasma glucose and insulin status. *Journal of Animal Science* **67**, 682-688.
131. Gore, D.C., Jahoor, F., Hibbert, J.M., and DeMaria E.J. (1996). Lactic acidosis during sepsis is related to increased pyruvate production, not deficits in tissue oxygen availability. *Annals of Surgery* **224**, 97-102.
132. Govoni, K.E., Hoagland, T.A., and Zinn, S.A. (2004). The ontogeny of the somatotropic axis in Hereford calves from birth to one year of age and its response to administration of exogenous bovine somatotropin. *Journal of Animal Science* **82**, 1646-1655.
133. Grimaldi, P.A., Teboul, L., Inadera, H., Gaillard, D., and Amri, E.Z. (1997). Transdifferentiation of myoblasts to adipoblasts: Triggering effects of fatty acids and thiazolidinediones. *Prostaglandins, Leukotrienes, and Essential Fatty Acids* **57,** 71–75.
134. Gross, R., and Mialhe, P. (1982). Beta adrenergic receptors mediated effects, insulin and plasma free fatty acids in the duck. *Hormone and Metabolic Research* **14**, 18-25.
135. Grygiel-Górniak, B. (2014). Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications – A review. *Nutrition Journal* **13**, 17.
136. Guest, S.J., Hadcock, J.R., Watkins, D.C., and Malbon, C.C. (1990). β_1- and β_2-adrenergic receptor expression in differentiating 3T3-L1 cells. Independent regulation at the level of mRNA. *The Journal of Biological Chemistry* **265**, 5370–5375.
137. Guntur, A.R., and Rosen, C.J. (2013). IGF-1 regulation of key signaling pathways in bone. *BoneKEy Reports* **2**, 437.
138. Gustafsson, M.K., Pan, H., Pinney, D.F., Liu, Y., Lewandowski, A., Epstein, D.J., and Emerson, C.P. Jr. (2002). Myf5 is a direct target of long-range Shh signaling and Gli regulation for muscle specification. *Genes and Development* **16**, 114-126.
139. Haemmerle, G., Lass, A., Zimmermann, R., Gorkiewicz, G., Meyer, C., Rozman, J., Heldmaier, G., Maier, R., Theussl, C., Eder, S., Kratky, D., Wagner, E.F., Klingenspor, M., Hoefler, G., and Zechner, R. (2006). Defective

lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science* **312**, 734-737.

140. Haemmerle, G., Zimmermann, R., Hayn, M., Theussl, C., Waeg, G., Wagner, E., Sattler, W., Magin, T.M., Wagner, E.F., and Zechner, R. (2002). Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. *The Journal of Biological Chemistry* **277**, 4806-4815.
141. Haffner, C.A., and Kendall, M.J. (1992). Metabolic effects of β_2-agonists. *Journal of Clinical Pharmacy and Therapeutics* **17**, 155-164.
142. Hagström-Toft, E., Enoksson, S., Moberg, E., Bolinder, J., and Arner, P. (1998). β-adrenergic regulation of lipolysis and blood flow in human skeletal muscle *in vivo*. *The American Journal of Physiology* **275**, E909-E916.
143. Hakonarson, H., and M. Grunstein. (1998). Regulation of second messengers associated with airway smooth muscle contraction and relaxation. *American Journal of Respiratory and Critical Care Medicine* **158**, S115–S122.
144. Halsey, C.H.C., Weber, P.S., Reiter, S.S., Stronach, B.N., Bartosh, J.L., and Bergen, W.G. (2011). The effect of ractopamine hydrochloride on gene expression in adipose tissues of finishing pigs. *Journal of Animal Scien*ce **89**, 1011-1019.
145. Hamby, P.L., Stouffer, J.R., and Smith, S.B. (1986). Muscle metabolism and real-time ultrasound measurement of muscle and subcutaneous adipose tissue growth in lambs fed diets containing a beta-agonist. *Journal of Animal Science*. **63**, 1410-1417.
146. Hammond, J. (1932). *Growth and Development of Mutton Qualities in Sheep*. Oliver and Boyd, Edinburgh, Scotland.
147. Han, X.H., Jin, Y-R., Seto, M., and Yoon, J.K. (2011). A WNT/β-Catenin signaling activator, R-spondin, plays positive regulatory roles during skeletal myogenesis. *The Journal of Biological Chemistry* **286**, 10649-10659.
148. Hansen, L.H., Madsen, B., Teisner, B., Nielsen, J.H., Billestrup, N. (1998). Characterization of the inhibitory effect of growth hormone on primary preadipocyte differentiation. *Molecular Endocrinology* **12**, 1140–1149
149. Hara, A., and Radin, N. (1978). Lipid extraction of tissues with low-toxicity solvent. *Analytical Biochemistry* **90**, 420–426.
150. Harris, D.M., Dunshea, F.R., Bauman, D.E., Boyd, R.D., Wang, S.Y., Johnson, P.A., and Clarke, S.D. (1993). Effect of *in vivo* somatotropin treatment of

growing pigs on adipose tissue lipogenesis. *Journal of Animal Science* **71**, 3293-3300.

151. Harrison, B.C., Allen, D.L., and Leinwand, L.A. (2011) IIb or not IIb? Regulation of myosin heavy chain gene expression in mice and men. *Skelet Muscle* **1**, 1–9.
152. Hasty, P., Bradley, A., Morris, J.H., Edmondson, D.G., Venuti, J.M., Olson, E.N., and Klein, W.H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**, 501–506.
153. Haunerland, N.H., and Spener, F. (2004). Fatty acid-binding proteins--insights from genetic manipulations. *Progress in Lipid Research* **43**, 328-349.
154. Hausman, G.J., and Martin, R.J. (1989). The influence of human growth hormone on preadipocyte development in serum-supplemented and serum-free cultures of stromal-vascular cells from pig adipose tissue. *Domestic Animal Endocrinology* **6**, 331-337.
155. Hausman, G.J., Dodson, M.V., Ajuwon, K., Azain, M., Barnes, K.M., Guan, L.L., Jiang, Z., Poulos, S.P., Sainz, R.D., Smith, S., Spurlock, M., Novakofski, J., Fernyhough, M.E., and Bergen, W.G. (2009). Board-invited review: The biology and regulation of preadipocytes and adipocytes in meat animals. *Journal of Animal Science* **87**, 1218-1246.
156. Hellerstein M.K. (1999). *De novo* lipogenesis in humans: Metabolic and regulatory aspects. *European Journal of Clinical Nutrition* **53**, S53-S65.
157. Hemmings, K.M., Daniel, Z.C.T.R., Buttery, P.J., Parr, T., and Brameld, J.M. (2015). Differential effects of short-term β-agonist and growth hormone treatments on expression of myosin heavy chain IIB and associated metabolic genes in sheep muscle. *Animal* **9**, 285–294.
158. Herrington, J., and Carter-Su, C. (2001). Signaling pathways activated by the growth hormone receptor. *Trends in Endocrinology and Metabolism* **12**, 252-257.
159. Hertzel, A.V., and Bernlohr, D.A. (2000). The mammalian fatty acid-binding protein multigene family: Molecular and genetic insights into function. *Trends in Endocrinology and Metabolism* **11**, 175-180.
160. Hinkle, R.T., Hodge, K.M., Cody, D.B., Sheldon, R.J., Kobilka, B.K., and Isfort, R.J. (2002). Skeletal muscle hypertrophy and anti-atrophy effects of

clenbuterol are mediated by the β_2-adrenergic receptor. *Muscle and Nerve* **25**, 729–734.

161. Hocquette, J., Gondret, F., Baéza, E., Médale, F., Jurie, C., & Pethick, D.W. (2010). Intramuscular fat content in meat-producing animals: Development, genetic and nutritional control, and identification of putative markers. *Animal* **4**, 303-319.
162. Hocquette, J.F., C. Jurie, Y. Ueda, P. Boulesteix, D. Bauchart & D.W. Pethick, (2003). The relationship between muscle metabolic pathways and marbling of beef. *In: Progress in Research on Energy and Protein Metabolism* Souffrant, W.B., Metges, C.C. (editors). EAAP Publication N° 109, Wageningen Pers., Wageningen, The Netherlands, 513-516.
163. Hogan, J.C., and Stephens, J.M. (2005). The regulation of fatty acid synthase by STAT5A. *Diabetes* **54**, 1968-1975.
164. Hollenberg, C.H., Raben, M.S., and Astwood, E.B. (1961). The lipolytic response to corticotropin. *Endocrinology* **68**, 589–598.
165. Holm, C., Osterlund, T., Laurell, H., and Contreras, J.A. (2000). Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annual Review of Nutrition* **20**, 365-393.
166. Hu, C.Y., Suryawan, A., Forsberg, N.E., Dalrymple, R.H., and Ricks, C.A. (1988). Effect of cimaterol on sheep adipose tissue lipid metabolism. *Journal of Animal Science* **66**, 1393-400.
167. Hu, E., Tontonoz, P., and Spiegelman, B.M. (1995). Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 9856-9860.
168. Hunter, J.G., van Delft, M.F., Rachubinski, R.A., and Capone, J.P. (2001). Peroxisome proliferator-activated receptor gamma ligands differentially modulate muscle cell differentiation and MyoD gene expression via peroxisome proliferator-activated receptor gamma-dependent and -independent pathways. *The Journal of Biological Chemistry* **276**, 38297-306.
169. Imanaka, M., Iida, K., Murawaki, A., Nishizawa, H., Fukuoka, H., Takeno, R., Takahashi, Y., Okimura, Y., Kaji, H., and Chihara K. (2008). Growth hormone stimulates mechano growth factor expression and activates myoblast

transformation in C2C12 cells. *The Kobe Journal of Medical Sciences* **54**, E46-E54.

170. Ingle, D.L., Bauman, D.E., and Garrigus, U.S. (1972). Lipogenesis in the ruminant: *In vivo* site of fatty acid synthesis in sheep. *The Journal of Nutrition* **102**, 617–624.
171. Jeong, J.Y., Jeoung, N.H., Park, K.-G., and Lee, I.-K. (2012). Transcriptional regulation of pyruvate dehydrogenase kinase. *Diabetes & Metabolism Journal* **36**, 328–335.
172. Ji, S., Doumit, M.E., and Hill, R.A. (2015). Regulation of adipogenesis and key adipogenic gene expression by 1,25-dihydroxyvitamin D in 3T3-L1 cells. *PLoS One* **10**, e0134199.
173. Jin, W., Peng, J., and Jiang, S. (2016). The epigenetic regulation of embryonic myogenesis and adult muscle regeneration by histone methylation modification. *Biochemistry and Biophysics Reports* **6**, 209–219.
174. Jocken, J.W.E., Smit E., Goossens, G.H., Essers, Y.P.G., van Baak, M.A., Mensink, M., Saris, W.H.M., and Blaak, E.E. (2008). Adipose triglyceride lipase (ATGL) expression in human skeletal muscle is type I (oxidative) fiber specific. *Histochemistry and Cell Biology* **129**, 535–538.
175. Johnson, B.J., Smith, S.B., and Chung, K.Y. (2014). Historical overview of the effect of β-adrenergic agonists on beef cattle production. *Asian-Australasian Journal of Animal Sciences* **27**, 757–766.
176. Johnson, M. (2006). Molecular mechanisms of β_2-adrenergic receptor function, response, and regulation. *Journal of Allergy and Clinical Immunology* **117**, 18-24.
177. José, A.A.F.B.V., Alvares, L.E., Delgado, E.F., Coutinho, L.L., and Lanna, D.P.D. (2006). Effect of growth hormone on fatty acid synthase gene expression in porcine adipose tissue cultures. *Genetics and Molecular Biology* **29**, 180-186.
178. Jurie, C., Cassar-Malek, I., Bonnet, M., Leroux, C., Bauchart, D., Boulesteix, P., Pethick, D.W., and Hocquette, J.F. (2007). Adipocyte fatty acid-binding protein and mitochondrial enzyme activities in muscles as relevant indicators of marbling in cattle. *Journal of Animal Science* **85**, 2660-2669.

179. Kadowaki, T., Yamauchi, T., and Kubota, N. (2008). The physiological and pathophysiological role of adiponectin and adiponectin receptors in the peripheral tissues and CNS. *FEBS Letters* **582**, 74-80

180. Kameyama, T., and Etlinger, J.D. (1979). Calcium-dependent regulation of protein synthesis and degradation in muscle. *Nature* **279**, 344-346.

181. Katafuchi, T., Garbers, D.L. and Albanesi, J.P. (2010). CNP/GC-B system: A new regulator of adipogenesis. *Peptides* **31**, 1906-1911.

182. Katiyar, S.S., Cleland, W.W., and Porter, J.W. (1975). Fatty acid synthetase. A steady state kinetic analysis of the reaction catalyzed by the enzyme from pigeon liver. *The Journal of Biological Chemistry* **250**, 2709-2717.

183. Kawai, M., Namba, N., Mushiake, S., Etani, Y., Nishimura, R., Makishima, M., and Ozono, K. (2007). Growth hormone stimulates adipogenesis of 3T3-L1 cells through activation of the Stat5A/5B-PPARγ pathway. *Journal of Molecular Endocrinology* **38**, 19-34.

184. Kawasaki, E., Hokari, F., Sasaki, M., Sakai, A., Koshinaka, K., and Kawanaka, K. (2011). The effects of β-adrenergic stimulation and exercise on NR4A3 protein expression in rat skeletal muscle. *The Journal of Physiological Sciences* **61**, 1–11.

185. Kazala, E.C., Petrak, J.L., Lozeman, F.J., Mir, P.S., Laroche, A., Deng, J. and Weselake, R.J. (2003). Hormone-sensitive lipase activity in relation to fat content of muscle in Wagyu hybrid cattle. *Livestock Production Science* **79**, 87-96.

186. Kershaw, E.E., Hamm, J.K., Verhagen, L.A., Peroni, O., Katic, M., and Flier, J.S. (2006). Adipose triglyceride lipase: Function, regulation by insulin, and comparison with adiponutrin. *Diabetes* **55**, 148-57.

187. Kersten S. (2001). Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Reports* **2**, 282–286.

188. Keyzer, M.A., Merbis, M.D., Pavel, I.F.P.W., and van Wesenbeeck, C.F.A. (2005). Diet shifts towards meat and the effects on cereal use: Can we feed the animals in 2030? *Ecological Economics* **55**, 187-202.

189. Khalfallah, Y., Sassolas, G., Borson-Chazot, F., Vega, N., and Vidal, H. (2001). Expression of insulin target genes in skeletal muscle and adipose tissue in adult patients with growth hormone deficiency: Effect of one year

recombinant human growth hormone therapy. *The Journal of Endocrinology* **171**, 285-292.

190. Kim, D.S., Itoh, E., Iida, K., and Thorner, M.O. (2008). Growth hormone increases mRNA levels of PPARδ and Foxo1 in skeletal muscle of growth hormone deficient lit/lit mice. *Endocrinology Journal* **56**, 141–147.
191. Kim, J.Y., Tillison, K., Lee, J.H., Rearick, D.A., and Smas, C.M. (2006). The adipose tissue triglyceride lipase ATGL/PNPLA2 is downregulated by insulin and TNF-α in 3T3-L1 adipocytes and is a target for transactivation by PPARγ. *American Journal of Physiology. Endocrinology and Metabolism* **291**, E115-E127.
192. Kim, S.P., Ha, J.M., Yun, S.J, Kim, E.K., Chung, S.W., Hong, K.W., Kim, C.D., and Bae, S.S. (2010). Transcriptional activation of peroxisome proliferator-activated receptor-gamma requires activation of both protein kinase A and Akt during adipocyte differentiation. *Biochemical and Biophysical Research Communications* **399**, 55-59.
193. Kim, Y.S., and Sainz, R.D. (1992). β-adrenergic agonists and hypertrophy of skeletal muscles. *Life Sciences* **50**, 397-407.
194. Knudtzon, J. (1984). Adrenergic effects on plasma levels of glucagon, insulin, glucose and free fatty acids in rabbits--influences of selective blocking drugs. *Acta Physiologica Scandinavica* **120**, 353-361.
195. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**, 656-660.
196. Koohmaraie, M., Shackelford, S.D., Muggli-Cockett, N.E., and Stone, R.T. (1991). Effect of the β-adrenergic agonist L-644,969 on muscle growth, endogenous proteinase activities, and postmortem proteolysis in wether lambs. *Journal of Animal Science* **69**, 4823-4835.
197. Kopchick, J.J, and Andry, J.M. (2000). Growth hormone (GH), GH receptor, and signal transduction. *Molecular Genetics and Metabolism* **71**, 293–314.
198. Kotzka, J., Knebel, B., Avci, H., Jacob, S., Nitzgen, U., Jockenhovel, F., Heeren, J., Haas, J., Muller-Wieland, D. (2010). Phosphorylation of sterol regulatory element-binding protein (SREBP)-1a links growth hormone action to lipid metabolism in hepatocytes. *Atherosclerosis* **213**, 156-65

199. Kraemer, F.B., and Shen, W-J. (2002). Hormone-sensitive lipase: Control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis. *Journal of Lipid Research* **43**, 1585–1594.
200. Krusinová, E., and Pelikánová T. (2008). Fatty acid binding proteins in adipose tissue: a promising link between metabolic syndrome and atherosclerosis? *Diabetes Research and Clinical Practice* **15**, S127-S134.
201. Labar, G., Wouters, J., and Lambert, D.M. (2010). A review on the monoacylglycerol lipase: At the interface between fat and endocannabinoid signalling. *Current Medicinal Chemistry* **17**, 2588-2607.
202. Ladeira, M.M., Schoonmaker, J.P., Gionbelli, M.P., Dias, J.C., Gionbelli, T.R., Carvalho, J.R., and Teixeira, P.D. (2016). Nutrigenomics and Beef Quality: A Review about Lipogenesis. *International Journal of Molecular Sciences* **1**, 918.
203. Lai, E., Rosen, O.M., and Rubin, C.S. (1982). Dexamethasone regulates the β-adrenergic receptor subtype expressed by 3T3-L1 preadipocytes and adipocytes. *The Journal of Biological Chemistry* **257**, 6691–6696.
204. Laliotis, G.P., Bizelis, I., and Rogdakis, E. (2010). Comparative approach of the *de novo* fatty acid synthesis (lipogenesis) between ruminant and non ruminant mammalian species: From biochemical level to the main regulatory lipogenic genes. *Current Genomics* **11**, 168–183.
205. Langfort, J., Ploug, T., Ihlemann, J., Saldo, M., Holm, C., and Galbo, H. (1999). Expression of hormone-sensitive lipase and its regulation by adrenaline in skeletal muscle. *The Biochemical Journal* **340**, 459-465.
206. Lanna, D.P.D., and Bauman, D.E. (1999) Effect of somatotropin, insulin, and glucocorticoid on lipolysis in chronic cultures of adipose tissue from lactating cows. *Journal of Dairy Science* **82**, 60-68.
207. Lanna, D.P.D., Houseknecht, K.L., Harris, D.M., and Bauman, D.E. (1995). Effects of somatotropin treatment on lipogenesis, lipolysis and related cellular mechanisms in adipose tissue of lactating cows. *Journal of Dairy Science* **78**, 1703-1712.
208. Larman, M.G., Katz-Jaffe, M.G., McCallie, B., Filipovits, J.A., and Gardner, D.K. (2011). Analysis of global gene expression following mouse blastocyst cryopreservation. *Human Reproduction* **26**, 2672-2680.

209. Laron, Z. (2001). Insulin-like growth factor 1 (IGF-1): A growth hormone. *Molecular Pathology* **54**, 311-316.

210. Lass, A., Zimmermann, R., Haemmerle, G., Riederer, M., Schoiswohl, G., Schweiger, M., Kienesberger, P., Strauss, J.G., Gorkiewicz, G., and Zechner, R. (2006). Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. *Cell Metabolism* **3,** 309-319.

211. Lass, A., Zimmermann, R., Oberer, M., and Zechner, R. (2011). Lipolysis – A highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. *Progress in Lipid Research* **50**, 14–27.

212. Laurell, H., Iacovoni, J.S., Abot, A., Svec, D., Maoret, J.-J., Arnal, J.-F., and Kubista, M. (2012). Correction of RT–qPCR data for genomic DNA-derived signals with ValidPrime. *Nucleic Acids Research* **40**, e51.

213. Lawrence, J.D., Mintert, J., Anderson, J.D., and Anderson, D.P. (2008). Feed grains and livestock: Impacts on meat supplies and prices. *American Agricultural Economics Association*. **23**.

214. Lawson, M.A., and Purslow, P.P. (2000). Differentiation of myoblasts in serum-free media: effects of modified media are cell line-specific. *Cells, Tissues, Organs* **167**, 130-137.

215. Lee, S.H., Joo, S.T., and Ryu Y.C. (2010). Skeletal muscle fiber type and myofibrillar proteins in relation to meat quality. *Meat Science* **86**, 166–170.

216. Lengyel, A.M.J. (2006). From growth hormone-releasing peptides to ghrelin: discovery of new modulators of GH secretion. *Arq Bras Endocrinol Metab* **50**, 17-24.

217. Levy, B., Desebbe, O., Montemont, C., and Gibot, S. (2008). Increased aerobic glycolysis through β_2 stimulation is a common mechanism involved in lactate formation during shock states. *Shock* **30**, 417-421.

218. Li, C.H., Simpson, M.E., and Evans, H.M. (1949). Influence of growth and adrenocorticotropic hormones on fat content of liver. *Archives of Biochemistry* **23**, 51-54.

219. Li, J.B., and Jefferson, L.S. (1977). Effects of isoproterenol on amino acid level and protein turnover in skeletal muscle. *The American Journal of Physiology* **232**, E243-E249.

220. Li, W.-C., Yu, W.-Y., Quinlan, J.M., Burke, Z.D. and Tosh, D. (2005). The molecular basis of transdifferentiation. *Journal of Cellular and Molecular Medicine* **9**, 569–582.
221. Listrat, A., Lebret, B., Louveau, I., Astruc, T., Bonnet, M., Lefaucheur, L., Picard, B., and Bugeon, J. (2016). How muscle structure and composition influence meat and flesh quality. *The Scientific World Journal* **2016**, 1–14.
222. Liu, C.Y., and Mills, S.E. (1990). Decreased insulin binding to porcine adipocytes *in vitro* by beta-adrenergic agonists. *Journal of Animal Science* **68**, 1603-1608.
223. Liu, C.Y., Grant, A.L., Kim, K.H., and Mills, S.E. (1994). Porcine somatotropin decreases acetyl-CoA carboxylase gene expression in porcine adipose tissue. *Domestic Animal Endocrinology* **11**, 125-132.
224. Liu, G., Li, X., and Godbout, R. (2008). A novel fatty acid-binding protein (FABP) gene resulting from tandem gene duplication in mammals: transcription in rat retina and testis. *Genomics* **92**, 436–445.
225. Liu, W., Thomas, S.G., Asa, S.L., Gonzalez-Cadavid, N., Bhasin, S., and Ezzat, S. (2003). Myostatin is a skeletal muscle target of growth hormone anabolic action. *The Journal of Clinical Endocrinology and Metabolism* **88**, 5490-5496.
226. Liu, Y., Ma, T., Du, W., Hao, H., Wang, D., Zhao, X., Li, H., Jiang, Q. and Zhu, H. (2012). Genes expression related to intramuscular fat deposition muscle of small tail Han sheep. *Journal of Animal and Veterinary Advances*. **11**, 3969-3977.
227. Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., and Darnell, J. (2000). *Molecular Cell Biology, 4th edition*. New York, NY. W. H. Freeman and Company.
228. Lonnqvist, F., Krief, S., Strosberg, A.D., Nyberg, S., Emorine, L.J., and Arner, P. (1993). Evidence for a functional β_3-adrenoceptor in man. *British Journal of Pharmacology* **110**, 929–936.
229. Louis, S.N.S., Jackman, G.P., Nero, T.L., Iakovidis, D., and Louis, W.J. (2000). Role of β-adrenergic receptor subtypes in lipolysis. *Cardiovascular Drugs and Therapy* **14**, 565-577.
230. Lum, L., and Beachy, P.A. (2004). The Hedgehog response network: Sensors, switches, and routers. *Science* **304,** 1755-1759.

231. Lynch, C.J., and Adams, S.H. (2014). Branched-chain amino acids in metabolic signalling and insulin resistance. *Nature Reviews. Endicronology* **10**, 723-736.
232. Lynch, G.S. and Ryall, J.G. (2008). Role of β-adrenoceptor signaling in skeletal muscle: Implications for muscle wasting and disease. *Physiological Reviews* **88**, 729-767.
233. Machida, S., and Booth, F.W. (2004). Insulin-like growth factor 1 and muscle growth: Implication for satellite cell proliferation. *Proceedings of the Nutrition Society* **63**, 337-340.
234. Maeda, K., Cao, H., Kono, K., Gorgun, C.Z., Furuhashi, M., Uysal, K.T., Cao, Q., Atsumi, G., Malone, H., Krishnan, B., Minokoshi, Y., Kahn, B.B., Parker, R.A., Hotamisligil, G.S. (2005). Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes. *Cell Metabolism* **1**, 107–119.
235. Magri, K.A., Adamo, M., Leroith, D., and Etherton, T.D. (1990). The inhibition of insulin action and glucose metabolism by porcine growth hormone in porcine adipocytes is not the result of any decrease in insulin binding or insulin receptor kinase activity. *Biochemical Journal* **266**, 107-113.
236. Mallette, L.E., Exton, J.H., and Park, C.R. (1969). Control of gluconeogenesis from amino acids in the perfused rat liver. *The Journal of Biological Chemistry* **244**, 5713–5723.
237. Maltin, C.A., Delday, M.I., Baillie, A.G.S., Grubb, D.A., and Garlick, P.J. (1989). Fiber-type composition of rat muscles. I. Changes during the first year of life. *The American Journal of Physiology* **257**, E823-E827.
238. Marchello, J.A. and Dryden, F.D. (1968). While Ideas Differ.. Meat Quality Is Defined. *Progressive Agriculture in Arizona* **20**, 14-15.
239. Masters, J.R., and Stacey, G.N. (2007). Changing medium and passaging cell lines. *Nature Protocols* **2**, 2276-2284.
240. Matsakas, A., Mouisel, E., Amthor, H., and Patel, K. (2010). Myostatin knockout mice increase oxidative muscle phenotype as an adaptive response to exercise. *Journal of Muscle Research and Cell Motility* **31**, 111–125.
241. Mayr, B., and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nature Reviews. Molecular Cell Biology* **2**, 599-609.

242. McCain, J. (2013). The MAPK (ERK) pathway: Investigational combinations for the treatment of BRAF-mutated metastatic melanoma. *Pharmacy and Therapeutics* **38**, 96–108.
243. McDowell, G.H. (1983). Hormonal control of glucose homoeostasis in ruminants. *The Proceedings of the Nutrition Society* **42**, 149-167.
244. McDowell, G.H., Gooden, J.M., Leenanuruksa, D., Jois, M., and English, A.W. (1987). Effects of exogenous growth hormone on milk production and nutrient uptake by muscle and mammary tissues of dairy cows in mid-lactation. *Australian Journal of Biological Sciences* **40**, 295-306.
245. McElligott, M.A., Barreto, A. Jr., and Chaung, L.Y. (1989). Effect of continuous and intermittent clenbuterol feeding on rat growth rate and muscle. *Comparative Biochemistry and Physiology. C, Comparative Pharmacology and Toxicology* **92**, 135-138.
246. McMeekan, C.P. (1940). Growth and development in the pig, with special reference to carcass quality characteristics. II. The influence of the plane of nutrition on growth and development. *Journal of Agricultural Science* **30**, 387-443.
247. McMillan, D.N., Noble, B.S., and Maltin, C.A. (1992). The effect of the β-adrenergic agonist clenbuterol on growth and protein metabolism in rat muscle cell cultures. *Journal of Animal Science* **70**, 3014-3023.
248. McPhee, M.J., Hopkins, D.L. and Pethick, D.W. (2008). Intramuscular fat levels in sheep muscle during growth. *Australian Journal of Experimental Agriculture* **48**, 904-909.
249. Mero, A.A., Ojala, T., Hulmi, J.J., Puurtinen, R., Karila, T.A., and Seppälä, T. (2010). Effects of alfa-hydroxy-isocaproic acid on body composition, DOMS and performance in athletes. *Journal of the International Society of Sports Nutrition* **7**, 1.
250. Mersmann, H.J. (1998). Overview of the effects of β-adrenergic receptor agonists on animal growth including mechanisms of action. *Journal of Animal Science* **76**, 160-172.
251. Mersmann, H.J. (2002). β-adrenergic receptor modulation of adipocyte metabolism and growth. *Journal of Animal Science* **80**, E24–E29.

252. Mills, S.E., and Liu, C.Y. (1990). Sensitivity of lipolysis and lipogenesis to dibutyryl-cAMP and β-adrenergic agonists in swine adipocytes *in vitro. Journal of Animal Science* **68**, 1017-1023.
253. Mills, S.E., Liu, C.Y., Gu, Y., and Schinckel, A.P. (1990). Effects of ractopamine on adipose tissue metabolism and insulin binding in finishing hogs. Interaction with genotype and slaughter weight. *Domestic Animal Endocrinology* **7**, 251-264.
254. Mizuno, T.M. (2015). β-hydroxypyruvate: A new diabetogenic factor? *Diabetes* **64**, 1099-1101.
255. Møller, N., and Jørgensen, J.O.L. (2009). Effects of growth hormone on glucose, lipid, and protein metabolism in human subjects. *Endocrine Reviews* **30**, 152-177.
256. Morales, P.E., Bucarey, J.L., and Espinosa, A. (2017). Muscle lipid metabolism: Role of lipid droplets and perilipins. *Journal of Diabetes Research* **2017**.
257. Moseti, D., Regassa, A., and Kim, W.-K. (2016). Molecular regulation of adipogenesis and potential anti-adipogenic bioactive molecules. *International Journal of Molecular Sciences* **17**, 124.
258. Mukherjee, R., Hoener, P.A., Jow, L., Bilakovics, J., Klausing, K., Mais, D.E., Faulkner, A., Croston, G.E., and Paterniti, J.R. (2000). A selective PPARγ modulator blocks adipocyte differentiation but stimulates glucose uptake in 3T3-L1 adipocytes. *Molecular Endocrinology* **14**, 1425–1433.
259. Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I., and Nabeshima, Y. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364**, 532–535.
260. Nader, G.A. (2005). Molecular determinants of skeletal muscle mass: Getting the "AKT" together. *The International Journal of Biochemistry and Cell Biology* **37**, 1985-1996.
261. Nam, S.Y., and Lobie, P.E. (2000). The mechanism of effect of growth hormone on preadipocyte and adipocyte function. *Obesity Reviews* **1**, 73-86.
262. National Research Council. (1988). *Understanding Agriculture: New Direction for Education.* The National Academies Press, Washington, D.C.

263. National Research Council. (1994). *Metabolic Modifiers: Effects on the Nutrient Requirements of Food-Producing Animals*. The National Academies Press, Washington, D.C.

264. Navegantes, L.C., Machado, C.R., Resano, N.M., Migliorini, R.H., and Kettelhut, I.C. (2003). β_2-agonists and cAMP inhibit protein degradation in isolated chick (*Gallus domesticus*) skeletal muscle. *British Poultry Science* **44**, 149-154.

265. Neve, B.P., Hoogerbrugge, N., Verhoeven, A.J., Birkenhäger, J.C., and Jansen, H. (1997). Growth hormone restores hepatic lipase mRNA levels but the translation is impaired in hepatocytes of hypothyroid rats. *Biochimica et Biophysica Acta* **1345**, 172-179.

266. Nonhebel, S. and Kastner, T. (2011). Changing demand for food, livestock feed and biofuels in the past and in the future. *Livestock Science* **139**, 3-10.

267. Nonogaki K. (2000). New insights into sympathetic regulation of glucose and fat metabolism. *Diabetologia* **43,** 533-549.

268. Norhazlin, J., Nor-Ashikin, M.N., Hoh, B.P., Sheikh Abdul Kadir, S.H., Norita, S., Mohd-Fazirul, M., Wan-Hafizah, W.J., Razif, D., Rajikin, M.H., and Abdullah, B. (2015). Effect of DNase treatment on RNA extraction from preimplantation murine embryos. *Genetics and Molecular Research* **14**, 10172-10184.

269. Norton, L.E., and Layman, D.K. (2006). Leucine regulates translation initiation of protein synthesis in skeletal muscle after exercise. *The Journal of Nutrition* **136**, 533S-537S.

270. O'Connor, R.M., Butler, W.R., Finnerty, K.D., Hogue, D.E., and Beermann, D.H. (1991). Acute and chronic hormone and metabolite changes in lambs fed the beta-agonist, cimaterol. *Domestic Animal Endocrinology* **8**, 537-548.

271. Okuda, H., Sekiya, K., and Nakamura, H. (1989). Lipolytic activities of p-aminophenol and n-decylamine in endogenous lipid droplets from rat adipocytes. *Pharmacological Research* **21**, 255-262.

272. Olaoye, O.A. (2011). Mini Review-Meat: An overview of its composition, biochemical changes and associated microbial agents. *International Food Research Journal* **18**, 877-885.

273. Olsson, B., Bohlooly-Y, M., Brusehed, O., Isaksson, O.G.P., Ahrén, B., Olofsson, S-O., Oscarsson, J., and Törnell, J. (2003). Bovine growth hormone-

transgenic mice have major alterations in hepatic expression of metabolic genes. *American Journal of Physiology - Endocrinology and Metabolism* **285**, E504-E511.

274. Orzechowski, A., Grzelkowska, K., Karlik, W., and Motyl, T. (2001). Eff ect of quercetin and DMSO on skeletal myogenesis from C2C12 skeletal muscle cells with special reference to PKB/Akt activity, myogenin and Bcl-2 expression. *Basic and Applied Myology* **11**, 31-44.
275. Oscai, L.B., Essig, D.A., and Palmer, W.K. (1990). Lipase regulation of muscle triglyceride hydrolysis. *Journal of Applied Physiology* **69**, 1571-1577.
276. Oscarsson, J., Ottosson, M., Vikman-Adolfsson, K., Frick, F., Enerbäck, S., Lithell, H., and Edén, S. (1999). GH but not IGF-I or insulin increases lipoprotein lipase activity in muscle tissues of hypophysectomised rats. *The Journal of Endocrinology* **160**, 247-255.
277. Ospina, P.A., McArt, J.A., Overton, T.R., Stokol, T., and Nydam, D.V. (2013). Using nonesterified fatty acids and β-hydroxybutyrate concentrations during the transition period for herd-level monitoring of increased risk of disease and decreased reproductive and milking performance. *The Veterinary Clinics of North America. Food Animal Practice* **29**, 387-412
278. Osuga, J., Ishibashi, S., Oka, T., Yagyu, H., Tozawa, R., Fujimoto, A., Shionoiri, F., Yahagi, N., Kraemer, F.B., Tsutsumi, O., and Yamada, N. (2000). Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 787-792.
279. Owens, P.C., Gatford, K.L, Walton, P.E., Morley, W., and Campbell, R. G. (1999). The relationship between endogenous insulin like growth factors and growth in pigs. *Journal of Animal Science* **77**, 2098-2103.
280. Page, K.A., Hartzell, D.L., Li, C., Westby, A.L., Della-Fera, M.A., Azain, M.J., Pringle, T.D., and Baile, C.A. (2004) β-Adrenergic receptor agonists increase apoptosis of adipose tissue in mice. *Domestic Animal Endocrinology* **26**, 23–31.
281. Pandini, G., Frasca, F., Mineo, R., Sciacca, L., Vigneri, R., and Belfiore, A. (2002). Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *The Journal of Biological Chemistry* **277**, 39684-39695.

282. Parr, T., Bardsley, R.G., Gilmour, R.S., and Buttery, P.J. (1992). Changes in calpain and calpastatin mRNA induced by βadrenergic stimulation of bovine skeletal muscle. *European Journal of Biochemistry* **208**, 333-339.

283. Patapoutian, A., Yoon, J.K., Miner, J.H., Wang, S., Stark, K., and Wold, B. (1995). Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development* **121**, 3347–3358.

284. Paul, R.J., and de Lanerolle, P. (1996). *Regulation of smooth muscle contractility. In Genetics of Asthma.* New York, NY. Marcel Dekker, Inc.

285. Pearen, M.A., Ryall, J.G., Maxwell, M.A., Ohkura, N., Lynch, G.S., and Muscat, G.E. (2006). The orphan nuclear receptor, NOR-1, is a target of β-adrenergic signaling in skeletal muscle. *Endocrinology* **147**, 5217-5227.

286. Pell, J., Elcock, C., Harding, R., Morrell, D., Simmonds, A., and Wallis, M. (1990). Growth, body composition, hormonal and metabolic status in lambs treated long-term with growth hormone. *British Journal of Nutrition*. **63**, 431-445.

287. Peterla, T.A., and Scanes, C.G. (1990). Effect of β-adrenergic agonists on lipolysis and lipogenesis by porcine adipose tissue *in vitro. Journal of Animal Science* **68**, 1024-1029.

288. Pethick, D.W., Harper, G.S., and Oddy, V.H. (2004). Growth, development and nutritional manipulation of marbling in cattle: A review. *Australian Journal of Experimental Agriculture* **44**, 705–715.

289. Pethick, D.W., Harper, G.S., Hocquette, J.F., and Wang, Y. (2006). Marbling biology – What do we know about getting fat into muscle? *Proceedings of Australian beef – the leader! The impact of science on the beef industry*, pp 103–110. CRC for Beef Genetic Technologies, University of New England, Armidale, NSW, Australia, 7–8 March 2006.

290. Pfaffl, M.W. (2005). *Nucleic Acids: mRNA identification and quantification.* In: Nucleic acids. Encyclopedia of analytical science. 2nd edn. (Worsfold PJ and Galagher PK, eds.). Academic Press, 417-426.

291. Philippou, A., Maridaki, M., Halapas, A., and Koutsilieris, M. (2007). The role of the insulin-like growth factor 1 (IGF-1) in skeletal muscle physiology. *In Vivo* **21**, 45-54.

292. Philipson, L.H. (2002). β-agonists and metabolism. *The Journal of Allergy and Clinical Immunology* **110,** S313-S317.

293. Philp, A., Belew, M.Y., Evans, A., Pham, D., Sivia, I., Chen, A., Schenk, S., and Baar, K. (2011). The PGC-1α-related coactivator promotes mitochondrial and myogenic adaptations in C2C12 myotubes. *American Journal of Physiology. Regulatory, Integrative, and Comparative Physiology* **301**, R864-R872.

294. Pocius, P.A., and Herbein, J.H. (1986). Effects of in vivo administration of growth hormone on milk production and in vitro hepatic metabolism in dairy cattle. *Journal of Dairy Science* **69**, 713-720.

295. Porte, D. (1967). Beta-adrenergic stimulation of insulin release in man. *Diabetes* **16**, 150-155.

296. Portiér, G.L., Benders, A.G., Oosterhof, A., Veerkamp, J.H., and van Kuppevelt, T.H. (1999). Differentiation markers of mouse C2C12 and rat L6 myogenic cell lines and the effect of the differentiation medium. *In Vitro Cellular and Developmental Biology. Animal* **35**, 219-227.

297. Postic, C., and Girard, J. (2008). Contribution of *de novo* fatty acid synthesis to hepatic steatosis and insulin resistance: Lessons from genetically engineered mice. *Journal of Clinical Investigation* **118**, 829-838.

298. Poulos, S.P. and Hausman, G.J. (2006). A comparison of thiazolidinedione-induced adipogenesis and myogenesis in stromal-vascular cells from subcutaneous adipose tissue or *semitendinosus* muscle of postnatal pigs. *Journal of Animal Science* **84**, 1076-1082.

299. Qian, S.W., Li, X., Zhang, Y.Y., Huang, H.Y., Li, Y., Sun, X., and Tang, Q.Q. (2010). Characterization of adipocyte differentiation from human mesenchymal stem cells in bone marrow. *BMC Developmental Biology* **10,** 47.

300. Qiao, Y., Huang, Z., Li, Q., Liu, Z., Hao, C., Shi, G., Dai, R., and Xie, Z. (2007). Developmental changes of the FAS and HSL mRNA expression and their effects on the content of intramuscular fat in Kazak and Xinjiang sheep. *Journal of Genetics and Genomics* **34**, 909-917.

301. Queipo-Ortuño, M.I., Escoté, X., Ceperuelo-Mallafré, V., Garrido-Sanchez, L., Miranda, M., Clemente-Postigo, M., Pérez-Pérez R., Peral, B., Cardona, F., Fernández-Real, J.M., Tinahones, F.J., and Vendrell J. (2012). FABP4 dynamics in obesity: Discrepancies in adipose tissue and liver expression regarding circulating plasma levels. *PLoS ONE* **7**, e48605.

302. Rahemi, H., Nigam, N., and Wakeling, J.M. (2015). The effect of intramuscular fat on skeletal muscle mechanics: Implications for the elderly and obese. *Journal of the Royal Society, Interface* **12**, 20150365.
303. Rajab, P., Fox, J., Riaz, S., Tomlinson, D., Ball, D., and Greenhaff, P.L. (2000). Skeletal muscle myosin heavy chain isoforms and energy metabolism after clenbuterol treatment in the rat. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **279**, R1076-R1081.
304. Rakhshandehroo, M., Sanderson, L.M., Matilainen, M., Stienstra, R., Carlberg, C., de Groot, P.J., Müller, M., and Kersten, S. (2007). Comprehensive analysis of PPARα-dependent regulation of hepatic lipid metabolism by expression profiling. *PPAR Research* **2007**, 26839.
305. Randle, P.J., Garland, P.B., Hales, C.N., and Newsholme, E.A. (1963). The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* **13**, 785-789.
306. Regmi, A., Deepak, M.S., Seale, J.L. and Bernstein, J. (2001). *Cross-Country Analysis of Food Consumption Patterns. Changing Structure of Global Food Consumption and Trade.* Anita Regmi, ed., Agriculture and Trade Report WRS-01-1, USDA, ERS, May, 2001.
307. Reichel, K., Rehfeldt, C., Weikard, R., Schadereit, R., and Krawielitzki, K. (1993). Effect of a β-agonist and a β-agonist/β-antagonist combination on muscle growth, body composition and protein metabolism in rats. *Archiv für Tierernaehrung* **45**, 211-225.
308. Reid, B.N., Ables, G.P., Otlivanchik, O.A., Schoiswohl, G., Zechner, R., Blaner, W.S., Goldberg, I.J., Schwabe, R.F., Chua, S.C., Jr., and Huang, L-S. (2008). Hepatic overexpression of hormone-sensitive lipase and adipose triglyceride lipase promotes fatty acid oxidation, stimulates direct release of free fatty acids, and ameliorates steatosis. *The Journal of Biological Chemistry* **283**, 13087–13099.
309. Reiter, S.S., Halsey, C.H., Stronach, B.M., Bartosh, J.L., Owsley, W.F., and Bergen, W.G. (2007). Lipid metabolism related gene-expression profiling in liver, skeletal muscle and adipose tissue in crossbred Duroc and Pietrain Pigs. *Comparative Biochemistry and Physiology, Part D Genomics and Proteomics* **2**, 200-206.

310. Relaix F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2005). A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* **435**, 948-953.

311. Rhinn, H., Marchand-Leroux, C., Croci, N., Plotkine, M., Scherman, D., Escriou, V. (2008). Housekeeping while brain's storming Validation of normalizing factors for gene expression studies in a murine model of traumatic brain injury. *BMC Molecular Biology* **9**, 62.

312. Richter, E.A., Ruderman, N.B., and Galbo, H. (1982). Alpha and beta adrenergic effects on metabolism in contracting, perfused muscle. *Acta Physiologica Scandinavica* **116**, 215-222.

313. Ricks, C.A., Dalrymple, R.H., Baker, P.K., and Ingle, D.L. (1984). Use of a β-agonist to alter fat and muscle deposition in steers. *Journal of Animal Science* **59**, 1247–1255.

314. Rohrer, D.K., Chruscinski, A., Schauble, E.H., Bernstein, D., and Kobilka, B.K. (1999). Cardiovascular and metabolic alterations in mice lacking both β_1- and β_2-adrenergic receptors. *The Journal of Biological Chemistry* **274**, 16701-16708.

315. Rosen, E.D., Walkey, C.J., Puigserver, P., and Spiegelman, B.M. (2000). Transcriptional regulation of adipogenesis. *Genes and Development* **14**, 1293–1307.

316. Rudnicki, M.A., Braun, T., Hinuma, S., and Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* **71**, 383-390.

317. Rudnicki, M.A., Schnegelsberg, P.N., Stead, R.H., Braun, T., Arnold, H.H., and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.

318. Ruiz-Canela, M., Toledo, E., Clish, C.B., Hruby, A., Liang, L., Salas-Salvadó, J., Razquin, C., Corella, D., Estruch, R., Ros, E., Fitó, M., Gómez-Gracia, E., Arós, F., Fiol, M., Lapetra, J., Serra-Majem, L., Martínez-González, M.A., and Hu, F.B. (2016). Plasma branched-chain amino acids and incident cardiovascular disease in the PREDIMED trial. *Clinical Chemistry* **62**, 582-592.

319. Russell-Jones, D. L. and Umpleby, A.M. (1999). *The effects of growth hormone on glucose & protein metabolism*. Growth Hormone. Boston, MA, Springer US: 163-172.
320. Ryall, J.G., Schertzer, J.D. and Lynch, G.S. (2008). Cellular and molecular mechanisms underlying age-related skeletal muscle wasting and weakness. *Biogerontology* **9**, 213–228.
321. Ryall, J.G., Sillence, M.N., and Lynch, G.S. (2006). Systemic administration of β_2-adrenoceptor agonists, formoterol and salmeterol, elicit skeletal muscle hypertrophy in rats at micromolar doses. *British Journal of Pharmacology* **147**, 587–595.
322. Ryan, K.J.P., Daniel, Z.C.T.R., Craggs, L.J.L., Parr, T., and Brameld, J.M. (2013). Dose-dependent effects of vitamin D on transdifferentiation of skeletal muscle cells to adipose cells. *The Journal of Endocrinology* **217**, 45–58.
323. Ryu, Y.C. and Kim, B.C. (2005). The relationship between muscle fibre characteristics, post-mortem metabolic rate, and meat quality of pig *longissimus dorsi* muscle. *Meat Science* **71**, 351-357.
324. Sabourin, L.A., and Rudnicki, M.A. (2000). The molecular regulation of myogenesis. *Clinical Genetics* **57**, 16-25.
325. Sakharova, A.A., Horowitz, J.F., Surya, S., Goldenberg, N., Harber, M.P., Symons, K., and Barkan, A. (2008). Role of growth hormone in regulating lipolysis, proteolysis, and hepatic glucose production during fasting. *Journal of Clinical Endocrinology and Metabolism* **93**, 2755-2759.
326. Sandouk, T., Reda, D., and Hofmann, C. (1993). Antidiabetic agent pioglitazone enhances adipocyte differentiation of 3T3-F442A cells. *The American Journal of Physiology* **264**, C1600-C1608.
327. Saponaro, C., Gaggini, M., Carli, F., and Gastaldelli, A. (2015). The subtle balance between lipolysis and lipogenesis: A critical point in metabolic homeostasis. *Nutrients* **7**, 9453–9474.
328. Sarjeant, K., & Stephens, J.M. (2012). Adipogenesis. *Cold Spring Harbor Perspectives in Biology* **4**, a008417.
329. Satterthwaite, D., McGranaham, G. and Tacoli, C. (2010). Urbanizations and its implications for food and farming. *Philosophical Transactions of the Royal Society B* **365**, 2809-2820.

330. Schachtrup, C., Emmler, T., Bleck, B., Sandqvist, A., and Spener, F. (2004). Functional analysis of peroxisome-proliferator-responsive element motifs in genes of fatty acid-binding proteins. *Biochemical Journal* **382**, 239-245.
331. Scharff, R., and Wool, I.G. (1966). Effect of diabetes on the concentration of amino acids in plasma and heart muscle of rats. *Biochemical Journal* **99**, 173–178.
332. Schiavetta, A.M., Miller, M.F., Lunt, D.K., Davis, S.K., Smith, S.B. (1990). Adipose tissue cellularity and muscle growth in young steers fed the β-adrenergic agonist clenbuterol for 50 days and after 78 days of withdrawal. *Journal of Animal Science* **68**, 3614–3623.
333. Schutz, Y. (2004). Concept of fat balance in human obesity revisited with particular reference to *de novo* lipogenesis. *International Journal of Obesity* **28**, S3–S11.
334. Scott, M.A., Nguyen, V.T., Levi, B. and James, A.W. (2011). Current methods of adipogenic differentiation of mesenchymal stem cells. *Stem Cells and Development* **20**, 1793-1804.
335. Scow, R.O., and Chernick, S.S. (1960). Hormonal control of protein and fat metabolism in the pancreatectomized rats. *Recent Progress in Hormone Research* **16**, 497-545.
336. Shavlakadze, T., Chai, J., Maley, K., Cozens, G., Grounds, G., Winn, N., Rosenthal, N., and Grounds, M.D. (2010). A growth stimulus is needed for IGF-1 to induce skeletal muscle hypertrophy *in vivo*. *Journal of Cell Science* **123**, 960-971.
337. Shen, C.N., Burke, Z.D., and Tosh, D. (2004). Transdifferentiation, metaplasia and tissue regeneration. *Organogenesis* **1**, 36-44.
338. Sheng, J., Vannela, R., and Rittmann, B.E. (2011). Evaluation of methods to extract and quantify lipids from Synechocystis PCC 6803. *Bioresource Technology* **102**, 1697–1703.
339. Siddle, K. (2011). Signalling by insulin and IGF receptors: Supporting acts and new players. *Journal of Molecular Endocrinology* **47,** R1-R10.
340. Siersbæk, R., Nielsen, R., and Mandrup, S. (2010). PPARγ in adipocyte differentiation and metabolism – Novel insights from genome-wide studies. *FEBS Letters*, **584,** 3242-3249.

341. Siersbæk, R., Nielsen, R., John, S., Sung, M.H., Baek, S., Loft, A., Hager, G.L., and Mandrup, S. (2011). Extensive chromatin remodelling and establishment of transcription factor "hotspots" during early adipogenesis. *EMBO Journal* **30**, 1459–1472.

342. Sillence, M.N., and Matthews, M.L. (1994). Classical and atypical binding sites for β-adrenoceptor ligands and activation of adenylyl cyclase in bovine skeletal muscle and adipose tissue membranes. *British Journal of Pharmacology* **111**, 866–872.

343. Sillence, M.N., Matthews, M.L., Spiers, W.G., Pegg, G.G., and Lindsay, D.B. (1991). Effects of clenbuterol, ICI118551 and sotalol on the growth of cardiac and skeletal muscle and on β_2-adrenoceptor density in female rats. *Naunyn-Schmiedeberg's Archives of Pharmacology* **344**, 449–453.

344. Singh, N.K., Chae, H.S., Hwang, I.H., Yoo, Y.M., Ahn, C.N., Lee, S.H., Lee, H.J., Park, H.J., and Chung, H.Y. (2007). Transdifferentiation of porcine satellite cells to adipoblasts with ciglitizone. *Journal of Animal Science* **85**, 1126-1135.

345. Sjögren, K., Bohlooly, Y.M., Olsson, B., Coschigano, K., Törnell, J., Mohan, S., Isaksson, O.G., Baumann, G., Kopchick, J., and Ohlsson C. (2000). Disproportional skeletal growth and markedly decreased bone mineral content in growth hormone receptor −/− mice. *Biochemical and Biophysical Research Communications* **267**, 603–608.

346. Smathers, R.L., and Petersen, D.R. (2011). The human fatty acid-binding protein family: Evolutionary divergences and functions. *Human Genomics* **5**, 170–191.

347. Smith, S.B. (1987). Effects of β-adrenergic agonists on cellular metabolism. *Reciprocal Meat Conference Proceedings*, **40**, 65-72.

348. Smith, S.B. (1991). *Biotechnology and animal products: probable effects on fat composition.*

349. Sohl, E., van Schoor, N.M., de Jongh, R.T., Visser, M., Deeg, D.J., and Lips, P. (2013). Vitamin D status is associated with functional limitations and functional decline in older individuals. *The Journal of Clinical Endocrinology and Metabolism* **98**, E1483-E1490.

350. Sotiropoulos, A., Ohanna, M., Kedzia, C., Menon, R.K., Kopchick, J.J., Kelly, P.A., and Pende, M. (2006). Growth hormone promotes skeletal muscle cell

fusion independent of insulin-like growth factor 1 up-regulation. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 7315–7320.

351. Spurlock, D.M., McDaneld, T.G., and McIntyre, L.M. (2006). Changes in skeletal muscle gene expression following clenbuterol administration. *BMC Genomics* **7**, 320.
352. Start, C., and Newsholme, E.A. (1968). Blood concentrations of ketone bodies and non-esterified fatty acids in starved and re- fed rats. *Biochemical Journal* **109**, 37P–38P.
353. Storch, J., and Thumser, A.E. (2010) Tissue-specific functions in the fatty acid-binding protein family. *The Journal of Biological Chemistry* **285**, 32679-32683.
354. Strydom, P.E., Frylinck, L., Montgomery, J.L., and Smith, M.F. (2009). The comparison of three β-agonists for growth performance, carcass characteristics and meat quality of feedlot cattle. *Meat Science* **81**, 557-564.
355. Sumantran, V.N., Tsai, M.L., Schwartz, J. (1992). Growth hormone induces c-fos and c-jun expression in cells with varying requirements for differentiation. *Endocrinology* **130**, 2016–2024.
356. Sun, L., Nicholson, A.C., Hajjar, D.P., Gotto, Jr. A.M., and Han, J. (2003). Adipogenic differentiating agents regulate expression of fatty acid binding protein and CD36 in the J744 macrophage cell line. *Journal of Lipid Research* **44**, 1877–1886.
357. Susulic, V.S., Frederich, R.C., Lawitts, J., Tozzo, E., Kahn, B.B., Harper, M.E., Himms-Hagen, J., Flier, J.S., and Lowell, B.B. (1995). Targeted disruption of the β_3-adrenergic receptor gene. *The Journal of Biological Chemistry* **270**, 29483-29492.
358. Svensson, R.U., Parker, S.J., Eichner, L.J., Kolar, M.J., Wallace, M., Brun, S.N., Lombardo, P.S., Van Nostrand, J.L., Hutchins, A., Vera, L., Gerken, L., Greenwood, J., Bhat, S., Harriman, G., Westlin, W.F., Harwood, H.J.Jr., Saghatelian, A., Kapeller, R., Metallo, C.M., and Shaw, R.J. (2016). Inhibition of acetyl-CoA carboxylase suppresses fatty acid synthesis and tumor growth of non-small-cell lung cancer in preclinical models. *Nature Medicine* **22**, 1108-1119.

359. Tanaka, T., Yoshida, N., Kishimoto, T., and Akira, S. (1997). Defective adipocyte differentiation in mice lacking the C/EBPβ and/or C/EBPδ gene. *EMBO Journal* **16**, 7432–7443.

360. Tang, B., Jeoung, D.I., and Sonenberg, M. (1995). Effect of human growth hormone and insulin on [3H] thymidine incorporation, cell cycle progression, and cyclin D expression in 3T3-F442A preadipose cells. *Endocrinology* **136**, 3062-3069.

361. Tavares, L., Alves, P.M., Ferreira, R.B., and Santos, C.N. (2011). Comparison of different methods for DNA-free RNA isolation from SK-N-MC neuroblastoma. *BMC Research Notes* **4**, 3.

362. Taylor, V.J., Beever, D.E. and Wathes, D.C. (2003). Physiological adaptations to milk production that affect fertility in high yielding dairy cows. *British Society of Animal Science Occasional Publication* **29**, 37–71.

363. Taylor-Jones, J.M., McGehee, R.E., Rando, T.A., Lecka-Czernik, B., Lipschitz, D.A., and Peterson, C.A. (2002). Activation of an adipogenic program in adult myoblasts with age. *Mechanism of Ageing and Development* **123**, 649-661.

364. Thornton, P.K. (2010). Livestock production: Recent trends, future prospects. *Philosophical Transactions of the Royal Society B* **365**, 2853-2867.

365. Tieland, M., Brouwer-Brolsma, E.M., Nienaber-Rousseau, C., van Loon, L.J., and De Groot, L.C. (2013). Low vitamin D status is associated with reduced muscle mass and impaired physical performance in frail elderly people. *European Journal of Clinical Nutrition* **67**, 1050-1055.

366. Tokach, R.J., Chung, K.Y., and Johnson, B.J. (2010). *Factors affecting intramuscular adipose tissue development in beef cattle*. Department of Animal and Food Sciences, Texas Tech University, Lubbock, 79409.

367. Tontonoz, P., and Spiegelman, B.M. (2008). Fat and beyond: the diverse biology of PPARγ. *Annual Review of Biochemistry* **77**, 289-312.

368. Torii, S.I., Kawada, T., Matsuda, K., Matsui, T., Ishihara, T., and Yano, H. (1998). Thiazolidinedione induces the adipose differentiation of fibroblast-like cells resident within bovine skeletal muscle. *Journal of Animal Science* **22**, 421-427.

369. Towle, H.C., Kaytor, E.N., and Shih, H.M. (1997). Regulation of the expression of lipogenic enzyme genes by carbohydrate. *Annual Review of Nutrition* **17**, 405-433.

370. Trepp, R., Flück, M., Stettler, C., Boesch, C., Ith, M., Kreis, R., Hoppeler, H., Howald, H., Schmid, J.P., Diem, P., and Christ, E.R. (2008). Effect of GH on human skeletal muscle lipid metabolism in GH deficiency. *American Journal of Physiology - Endocrinology and Metabolism* **294**, E1127-E1134.

371. Tsumaki, N., and Yoshikawa, H. (2005). The role of bone morphogenetic proteins in endochondral bone formation. *Cytokine and Growth Factor Reviews* **16**, 279-285.

372. Turner, P.R., Westwood, T., Regen, C.M., and Steinhardt, R.A. (1988). Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. *Nature* **335**, 735-738.

373. Tyagi, S., Gupta, P., Saini, A.S., Kaushal, C., and Sharma, S. (2011). The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. *Journal of Advanced Pharmaceutical Technology & Research* **2**, 236-240.

374. Underwood, K.R., Means, W.J., Zhu, M.J., Ford, S.P., Hess, B.W., and Du, M. (2008). AMP-activated protein kinase is negatively associated with intramuscular fat content in *longissimus dorsi* muscle of beef cattle. *Meat Science* **79**, 394-402.

375. Valin, H., Sands, R.D, van der Mensbrugghe, D., Nelson, G.C., Ahammad, H., Blanc, E., Bodirsky, B., Fujimori, S., Hasegawa, T., Havlik, P., Heyhoe, E., Kyle, P., Mason-D'Croz, D., Paltsev, S., Rolinski, S., Tabeau, A., van Meijl, H., von Lampe, M., and Willenbockel, D. (2014). The future of food demand: Understanding the differences in global economic models. *Agricultural Economics* **45**, 51-67.

376. van Amerongen, R., and Nusse, R. (2009). Towards an integrated view of Wnt signaling in development. *Development* **136**, 3205-3214.

377. van der Meijden, K., Bravenboer, N., Dirks, N.F., Heijboer, A.C., den Heijer, M., de Wit, G.M.J., Offringa, C., Lips, P. and Jaspers, R.T. (2016). Effects of $1,25(OH)_2D_3$ and $25(OH)D_3$ on C2C12 myoblast proliferation, differentiation, and myotube hypertrophy. *Journal of Cellular Physiology* **231**, 2517–2528.

378. van Laack, R.L., Stevens, S.G., and Stalder, K.J. (2001). The influence of ultimate pH and intramuscular fat content on pork tenderness and tenderization. *Journal of Animal Science* **79**, 392-397.
379. Vaughan, M., Berger, J.E., and Steinberg, D. (1964). Hormone-sensitive lipase and monoglyceride lipase activities in adipose tissue. *The Journal of Biological Chemistry* **239**, 401–409.
380. Velloso, C.P. (2008). Regulation of muscle mass by growth hormone and IGF-1. *British Journal of Pharmacology* **154**, 557-568.
381. Verghese, P.B., Arrese, E.L., and Soulages, J.L. (2007). Stimulation of lipolysis enhances the rate of cholesterol efflux to HDL in adipocytes. *Molecular and Cellular Biochemistry* **302**, 241-248.
382. Vernon, R.G. (1980). Lipid metabolism in the adipose tissue of ruminant animals. *Progress in Lipid Research* **19**, 23-106.
383. Vernon, R.G. (1981). Lipid metabolism in the adipose tissue of ruminants. *In: Lipid metabolism in Ruminant Animals* W.W. Christie (editor), Pergamon Press, Oxford, 279–362.
384. Vernon, R.G., Barber, M., and Finley, E. (1991). Modulation of the activity of acetyl-coA carboxylase and other lipogenic enzymes by growth hormone, insulin and dexamethasone in sheep adipose tissue and relationship to adaptations to lactation. *Biochemical Journal* **274**, 543–548.
385. Vestergaard, M., Henckel, P., Oksbjerg, N., and Sejrsen, K. (1994). The effect of cimaterol on muscle fiber characteristics, capillary supply, and metabolic potentials of longissimus and semitendinosus muscles from young Friesian bulls. *Journal of Animal Science* **72**, 2298-2306.
386. Vestergaard, M., Purup, S., Frystyk, J., Løvendahl, P., Sørensen, M.T, Riis, P.M., Flint, D.J., and Sejrsen, K. (2003). Effects of growth hormone and feeding level on endocrine measurements, hormone receptors, muscle growth and performance of prepubertal heifers. *Journal of Animal Science* **81**, 2189-2198.
387. Vijayakumar, A., Novosyadlyy, R., Wu, Y., Yakar, S., and LeRoith, D. (2010). Biological effects of growth hormone on carbohydrate and lipid metabolism. *Growth Hormone and IGF Research* **20**, 1-7.
388. Vijayakumar, A., Yakar, S., and Leroith, D. (2011). The intricate role of growth hormone in metabolism. *Frontiers in Endocrinology* **2**, 32.

389. Wabitsch, M., Heinze, E., Hauner, H., Shymko, R.M., Teller, W.M., De Meyts, P., and Ilondo, M.M. (1996). Biological effects of human growth hormone in rat adipocyte precursor cells and newly differentiated adipocytes in primary culture. *Metabolism* **45**, 34-42.

390. Wang, N.D., Finegold, M.J., Bradley, A., Ou, C.N., Abdelsayed, S.V., Wilde, M.D., Taylor, L.R., Wilson, D.R., and Darlington, G.J. (1995). Impaired energy homeostasis in C/EBPα knockout mice. *Science* **269**, 1108–1112.

391. Wang, Y., Bikle, D.D., and Chang, W. (2013). Autocrine and paracrine actions of IGF-I signaling in skeletal development. *Bone Research* **1**, 249-259.

392. Wang, Y., Fried, S.K., Petersen, R.N., and Schoknecht, P.A. (1999). Somatotropin regulates adipose tissue metabolism in neonatal swine. *The Journal of Nutrition* **129**, 139-145.

393. Wang, Y.H., Byrne, K.A., Reverter, A., Harper, G.S., Taniguchi, M., McWilliam, S.M., Mannen, H., Oyama, K., and Lehnert. S.A. (2005). Transcriptional profiling of skeletal muscle tissue from two breeds of cattle. *Mammalian Genome* **16**, 201–210.

394. Wang, Z., Masternak, M.M., Al-Regaiey, K.A., and Bartke, A. (2007). Adipocytokines and the regulation of lipid metabolism in growth hormone transgenic and calorie-restricted mice. *Endocrinology* **148**, 2845–2853.

395. Wannenes, F., Magni, L., Bonini, M., Dimauro, I., Caporossi, D., Moretti, C., and Bonini, S. (2012). *In vitro* effects of beta-2 agonists on skeletal muscle differentiation, hypertrophy, and atrophy. *The World Allergy Organization Journal* **5**, 66–72.

396. Ward, K.A., Das, G., Roberts, S.A., Berry, J.L., Adams, J.E., Rawer, R., and Mughal, M.Z. (2010). A randomized, controlled trial of vitamin D supplementation upon musculoskeletal health in postmenarchal females. *The Journal of Clinical Endocrinology and Metabolism* **95**, 4643-4651.

397. Ward, R.E., Woodward, B., Otter, N., and Doran, O. (2010). Relationship between the expression of key lipogenic enzymes, fatty acid composition, and intramuscular fat content of Limousin and Aberdeen Angus cattle. *Livestock Science* **127**, 22-29.

398. Warriss, P.D. (1990). The handling of cattle pre-slaughter and its effects on carcass and meat quality. *Applied Animal Behaviour Science* **28**, 171-186.

399. Warriss, P.D., Kestin, S.C., and Brown, S.N. (1989). The effect of beta-adrenergic agonists on carcass and meat quality in sheep. *Animal Production* **48**, 385-392.

400. Warriss, P.D., Kestin, S.C., Rolph, T.P., and Brown, S.N. (1990). The effects of the beta-adrenergic agonist salbutamol on meat quality in pigs. *Journal of Animal Science* **68,** 128-136.

401. Watson-Wright, W.M. and Wilkinson, M. (1986). The muscle slice - A new preparation for the characterization of β-adrenergic binding the fast and slow twitch skeletal muscle. *Muscle and Nerve* **9**, 416-422.

402. Watt, M.J., and Hoy, A.J. (2012). Lipid metabolism in skeletal muscle: generation of adaptive and maladaptive intracellular signals for cellular function. *American Journal of Physiology. Endocrinology and Metabolism* **302**, E1315-E1328.

403. Webb, E.C., and O'Neill, H.A. (2008): The animal fat paradox and meat quality. *Meat Science* **80**, 28-36.

404. Weber, T.E., Richert, B.T., Belury, M.A., Gu, Y., Enright, K., and Schinckel. A.P. (2006). Evaluation of the effects of dietary fat, conjugated linoleic acid, and ractopamine on growth performance, pork quality, and fatty acid profiles in genetically lean gilts. *Journal of Animal Science* **84**, 720-732.

405. Weinstock, P.H., Levak-Frank, S., Hudgins, L.C., Radner, H., Friedman, J.M., Zechner, R., and Breslow, J.L. (1997). Lipoprotein lipase controls fatty acid entry into adipose tissue, but fat mass is preserved by endogenous synthesis in mice deficient in adipose tissue lipoprotein lipase. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 10261-10266.

406. Wimmers, K., Ngu, N.T., Jennen, D.G.J., Tesfaye, D., Murani, E., Schellander, K., and Ponsuksili, S. (2008). Relationship between myosin heavy chain isoform expression and muscling in several diverse pig breeds. *Journal of Animal Science* **86**, 795–803.

407. Wood, J.D., Enser, M., Fisher, A.V., Nute, G.R., Sheard, P.R., Richardson, R.I., Hughes, S.I., and Whittington, F.M. (2008). Fat deposition, fatty acid composition and meat quality: A review. *Meat Science* **78**, 343-358.

408. Wray, C.J., Sun, X., Gang, G.I., and Hasselgren, P-O. (2002). Dantrolene downregulates the gene expression and activity of the ubiquitin-proteasome

proteolytic pathway in septic skeletal muscle. *Journal of Surgical Research* **104**, 82-87.

409. Wu, J., Liu, W., Liu, J., Qiao, L., and Yuan, Y. (2011). Distribution and quantification of β_3-adrenergic receptor in tissues of sheep. *Animal* **5**, 88-93.
410. Xiaomei, H., Yongsheng, H., Cai, H., Li, Y., Fang, M., Jia, X., Nie, Q., and Zhang, X. (2012). cDNA cloning and expression analysis of ATGL gene in four avian species. *Animal Science Papers and Reports*. **30**, 271-284.
411. Xu, C., Shu, S., Xia, C., Wang, B., Zhang, H.Y. (2014). Investigation on the relationship of insulin resistance and ketosis in dairy cows. *Journal of Veterinary Science Technology* **5**, 162.
412. Yan, X., Zhu, M.J., Dodson, M.V., and Du, M. (2013). Developmental programming of fetal skeletal muscle and adipose tissue development. *Journal of Genomics* **1**, 29-38.
413. Yang, S., Mulder, H., Holm, C., and Edén, S. (2004). Effects of growth hormone on the function of β-adrenoceptor subtypes in rat adipocytes. *Obesity Research* **12**, 330-339.
414. Yang, X., Lu, X., Lombès, M., Rha, G.B., Chi, Y.I., Guerin, T.M., Smart, E.J., and Liu, J. (2010b). The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. *Cell Metabolism* **11**, 194-205.
415. Yang, Y.T. and McElligott, M.A. (1989). Multiple actions of β-adrenergic agonists on skeletal muscle and adipose tissue. *Biochemical Journal* **261**, 1-10.
416. Yeh, W.C., Cao, Z., Classon, M., and McKnight, S.L. (1995). Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes and Development* **9**, 168–181.
417. Yeow, K., Phillips, B., Dani, C., Cabane, C., Amri, E.Z. and De'rijard, B, (2001). Inhibition of myogenesis enables adipogenic transdifferentiation in the C2C12 myogenic cell line. *FEBS Letters* **506**,157-162.
418. Yimlamai, T., Dodd, S.L., Borst, S.E., and Park, S. (1985). Clenbuterol induces muscle-specific attenuation of atrophy through effects on the ubiquitin-proteasome pathway. *Journal of Applied Physiology* **99**, 71-80.
419. Yin, D., Clarke S.D., Peters J.L., and Etherton. T.D. (1998). Somatotropin-dependent decrease in fatty acid synthase mRNA abundance in 3T3-F442A

adipocytes is the result of a decrease in both gene transcription and mRNA stability. *Biochemical Journal* **331**, 815–820.

420. Youssef, M. and M. El-Ashker. (2017). Significance of insulin resistance and oxidative stress in dairy cattle with subclinical ketosis during the transition period. *Tropical Animal Health and Production* **49**, 239-244.
421. Yu, K., Shu, G., Yuan, F., Zhu, X., Gao, P., Wang, S., Wang, L., Xi, Q., Zhang, S., Zhang, Y., Li, Y., Wu, T., Yuan, L. and Jiang, Q. (2013). Fatty acid and transcriptome profiling of *longissimus dorsi* muscles between pig breeds differing in meat quality. *International Journal of Biological Sciences* **9**, 108-118.
422. Zamboni M1, Zoico E, Tosoni P, Zivelonghi A, Bortolani A, Maggi S, Di Francesco V, Bosello O. (2002). Relation between vitamin D, physical performance, and disability in elderly persons. *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* **57**, M7-M11.
423. Zechner, R., Kienesberger, P.C., Haemmerle, G., Zimmermann, R., and Lass, A. (2009). Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *Journal of Lipid Research* **50**, 3-21.
424. Zechner, R., Zimmermann, R., Eichmann, T.O., Kohlwein, S.D., Haemmerle, G., Lass A., and Madeo, F. (2012). Fat signals - Lipases and lipolysis in lipid metabolism and signalling. *Cell Metabolism* **15**, 279-291.
425. Zeman, R.J., Kameyama, T., Matsumoto, K., Bernstein, P., and Etlinger, J.D. (1985). Regulation of protein degradation in muscle by calcium. Evidence for enhanced nonlysosomal proteolysis associated with elevated cytosolic calcium. *The Journal of Biological Chemistry* **260**, 13619-13624.
426. Zhang, L., Zhao, Y., Ning, Y., Wang, H., and Zan, L. (2017). Ectopical expression of FABP4 gene can induce bovine muscle-derived stem cells adipogenesis. *Biochemical and Biophysical Research Communications* **482**, 352–358.
427. Zhang, S., Hulver, M.W., McMillan, R.P., Cline, M.A., and Gilbert, E.R. (2014). The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. *Nutrition and Metabolism* **11**, 10.
428. Zhang, W., Behringer, R.R., and Olson, E.N. (1995). Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. *Genes and Development* **9**, 1388–1399.

429. Zhang, Y., Kobayashi, H., Mawatari, K., Sato, J., Bajotto, G., Kitaura, Y., and Shimomura, Y. (2011). Effects of branched-chain amino acid supplementation on plasma concentrations of free amino acids, insulin, and energy substrates in young men. *Journal of Nutritional Science and Vitaminology* **57**, 114-117.
430. Zhu, J.J., Luo, J., Wang, W., Yu, K., Wang, H.B., Shi, H.B., Sun, Y.T., Lin, X.Z., and Li, J. (2014). Inhibition of FASN reduces the synthesis of medium-chain fatty acids in goat mammary gland. *Animal* **8**, 1469-1478.
431. Zimmerman, A.W., and Veerkamp, J.H. (2002). New insights into the structure and function of fatty acid-binding proteins. *Cellular and Molecular Life Sciences* **59**, 1096–1116.
432. Zimmermann, R., Lass, A., Haemmerle, G., and Zechner, R. (2009). Fate of fat: The role of adipose triglyceride lipase in lipolysis. *Biochimica et Biophysica Acta* **1791**, 494-500.
433. Zimmermann, R., Strauss, J.G., Haemmerle, G., Schoiswohl, G., Birner-Gruenberger, R., Riederer, M., Lass, A., Neuberger, G., Eisenhaber, F., Hermetter, A., and Zechner, R. (2004). Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* **306**, 1383-1386.