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Functional proteome and microRNAome of beef cattle adipose tissue

by

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Dedication

I dedicate this work to the Lord, for never giving up on me and for showing the greatness of his compassionate love in my life. The Lord not only guided me to this wonderful opportunity of being a PhD student, but also put me together with best crew I could ever wish for accompanying me during this journey: my beloved wife and soul mate Thania Moraes, my inspiring and dedicated supervisor Dr. Leluo Guan, my loving and supportive parents Antonio Jesualdo and Maria Socorro, and great brothers Filipe Romão and Vinícius Romão.

Abstract

Adipose tissue plays an important role in the energetic balance, but it is also an important aspect of meat quality and animal productivity. However, the understanding on how fat metabolism is regulated in beef cattle is not clear. This research aimed at exploring how bovine adipose tissue is molecularly regulated by proteins and microRNAs (miRNAs) under different conditions. Study 1 detected a total of 244 miRNAs in subcutaneous and visceral adipose tissue of steers fed different diets (high or low fat content). Several miRNAs responded to dietary changes (miR-19a, -92a, -92b, -101, -103, -106, -142-5p, and 296) or fat depot (miR-196a and -2454), and were predicted to be involved in different aspects of lipid metabolism such as lipid synthesis and oxidation. Study 3 showed that the compositional structure of DNA that surrounded 155 miRNA genes was associated with miRNA expression and function as clustered miRNAs and highly conserved miRNAs were more highly expressed and had more predicted targets than non-clustered or less conserved miRNAs. Bovine miRNAs were shown to work collectively in regulatory networks with other miRNAs or with genes that host intronic miRNAs. Study 2 identified and quantified 682 proteins in bovine adipose tissue revealing that the proteomic profile differs between fat depots, indicating important functional and physiological differences such as a higher metabolic activity of visceral fat. Besides, fat depots also respond differently to diet, as subcutaneous fat seems to be more responsive to dietary changes. Study 4 showed that subcutaneous fat expansion in steers from 12 to 15 months of age

(finishing) occurred concomitant with expression changes in 123 out of 627 proteins. Differentially expressed (DE) proteins were associated with several biological processes. Proteomic changes related to lipid metabolism indicated a reduction in the synthesis of fatty acids at the cellular level when steers were older. These findings improved our understanding on how adipose tissue is regulated. They may help the development of strategies to manipulate bovine adiposity that consider the specific molecular regulation and physiology of different fat depots and their response to dietary manipulation, as well as the different periods of development of beef cattle.

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List of Abbreviations

ANOVA	Analysis of variance
AT	Adipose tissue
BAT	Brown adipose tissue
bta	*Bos taurus*
cDNA	Complementary DNA
C.SAT	Control group - subcutaneous adipose tissue
C.VAT	Control group - visceral adipose tissue
CV	Coefficient of variation
DE	Differentially expressed
FC	Fold change
FD	Fold difference
HF.SAT	High fat group - subcutaneous adipose tissue
HF.VAT	High fat group - visceral adipose tissue
IPA	Ingenuity pathway analysis
Kb	Kilo base, 1,000 bp
LC-MS	Liquid chromatography - mass spectrometry
LFQ	Label Free Quantification
Mb	Mega base, 1,000,000 bp
MSC	Mesenchymal stem cells
miRISC	microRNA induced silencing complex
miRNA	MicroRNA
mRNA	Messenger RNA

qRT-PCR	Quantitative reverse transcription polymerase chain reaction
R	Coefficient of correlation
R^2	Coefficient of determination
SD	Standard deviation
SV	Stromal vascular
UTR	Untranslated region
WAT	White adipose tissue

1 Literature review

1.1 INTRODUCTION

Meat is a fundamental source of protein for humans. Estimates indicate that the worldwide demand for meat is expected to grow 73% from 2010 to 2050 (FAO, 2011). Beef ranks as the third most important meat in consumption, behind only swine and poultry. Canada and especially Alberta have an important role in securing the beef supply as they are major producers and exporters of beef with more than 13 million cattle in 2012 (Statistics Canada, 2012). In this context, strategies to improve and increase beef production are fundamental to meet the demand for beef in the coming years. Adipose tissue is an important component in animal productivity and meat quality (Hausman et al., 2009; Dodson et al., 2010b). Intramuscular fat is responsible for marbling, which improves meat flavor. Manipulating (increasing) the amount of intramuscular fat is one of the goals for the beef industry to provide the consumer with a better product (Dodson et al., 2010b). At the same time it is critical to control the amount of fat in other fat depots that are normally not consumed, so as to prevent excess subcutaneous and visceral fat which would have a negative impact on animal productivity (Dodson et al., 2010b). Therefore, understanding the regulation of fat metabolism is fundamental to developing strategies to manipulate fat; however, our knowledge on the molecular mechanisms of bovine adipose tissue is very limited.

Adipose tissue plays an important role in the regulation of energy homeostasis, however this tissue was considered a metabolically inert tissue until the second half of the 20th century (Hirsch, 1984). Since the 50's, murine models

have been widely used to study obesity in order to better understand the regulation of adipose tissue in mammals (Hariri and Thibault, 2010). Studies in the past decades have unveiled the importance of adipose tissue, showing not only the role it plays in the regulation of fat and energy metabolism, but also as an endocrine organ releasing several adipokines (Poulos et al., 2010b). Another aspect that gathered the attention of researchers for adipose tissue was the significant increase of obesity in humans which is a critical risk for human health, especially in developed countries (Galic et al., 2010). Not only in humans, obesity is now a major concern of health in companion animals, such as dogs and cats which are being increasingly affected by this condition (German et al., 2010). Besides health aspects, adipose tissue has been studied in farm animals, especially those used for meat production such as beef cattle, pigs and broilers. This stems from the fact that adipose tissue is an important component of quality and nutritional value of the meat and can also impact animal productivity (Hausman et al., 2009). However, the understanding of adipogenesis in livestock species is very limited and the molecular mechanisms of bovine adipogenesis are yet to be discovered.

Adipogenesis is the process by which immature adipose cells differentiate into specialized mature cells called adipocytes. These cells play an important role in energy regulation as they are able to synthesize and store fatty acids in the form of triglycerides as well release free fatty acids through lipolysis (Large et al., 2004). Not only do adipose cells store and release fatty acids, but they also release a series of adipokines that regulate energy metabolism, such as leptin which inhibits food intake and stimulates energy expenditure (Galic et al., 2010).

Adipogenesis in mammals is regulated genetically. Studies have shown the importance of adipogenic transcription factors such as Peroxisome Proliferator Activated Receptor γ (PPARγ), CCAAT-Enhancer Binding Proteins (C/EBPs), Kruppel Like Factors (KLFs) and Sterol Regulatory Element Binding Protein (SREBP), which regulate the expression of many adipogenic genes involved in the differentiation of adipocytes (Lefterova and Lazar, 2009; White and Stephens, 2010). Besides transcriptional regulation, recent studies have shown that microRNAs (miRNAs), a family of small non coding RNAs with lengths of approximately 22 nucleotides, have been found to play a role in the regulation and development of adipocytes in humans and mice (Xie et al., 2009). MicroRNAs have been reported to participate in several biological processes including cell differentiation, animal development, metabolism, tumorigenesis and other diseases (Alvarez-Garcia and Miska, 2005; Wienholds and Plasterk, 2005; Krützfeldt and Stoffel, 2006; Lee et al., 2006; O'Rourke et al., 2006; Lynn, 2009). MicroRNAs have the ability to regulate gene expression by targeting messenger RNAs and promoting translational repression, mRNA degradation or mRNA sequestration (Williams, 2008). These previous studies suggest that adipogenesis is a complex process controlled by multiple regulatory mechanisms. However, defining the molecular mechanisms in adipogenesis and adipocyte physiology could be key to developing methods that optimizing meat quality and growth efficiency in beef cattle (Hausman et al., 2009).

1.2 FAT BIOLOGY [1]

1.2.1 Adipogenesis

Adipogenesis is the process by which non specialized stem cells turn into mature adipocytes as shown in Figure 1.1. This process can be divided in two stages: commitment and differentiation. Commitment is the first stage in which stem cells are converted into preadipocytes. The differentiation stage follows the commitment stage and converts the preadipocytes into mature adipocytes which are capable of secreting adipokines, transporting, synthesizing, and releasing lipids. The expansion of adipose tissue relies on hypertrophy (increase in tissue volume due to enlargement of cells) and hyperplasia (increase in tissue volume due to proliferation of cells). Our understanding on how adipogenesis works in mammals relies heavily on *in vitro* culture systems with the use of preadipocyte cell lines; mature adipocyte-derived dedifferentiated fat cells, mesenchymal stem cells (MSCs) and adipose-derived stem cells (Moreno-Navarrete and Fernández-Real, 2012).

[1] The Fat biology section is part of a book chapter accepted for publication: Romao, J.M. & Guan, LL. 2013, Adipogenesis and Obesity. In: MicroRNAs in Regenerative Medicine. Dr. Sen, C.K. (ed). ELSEVIER.

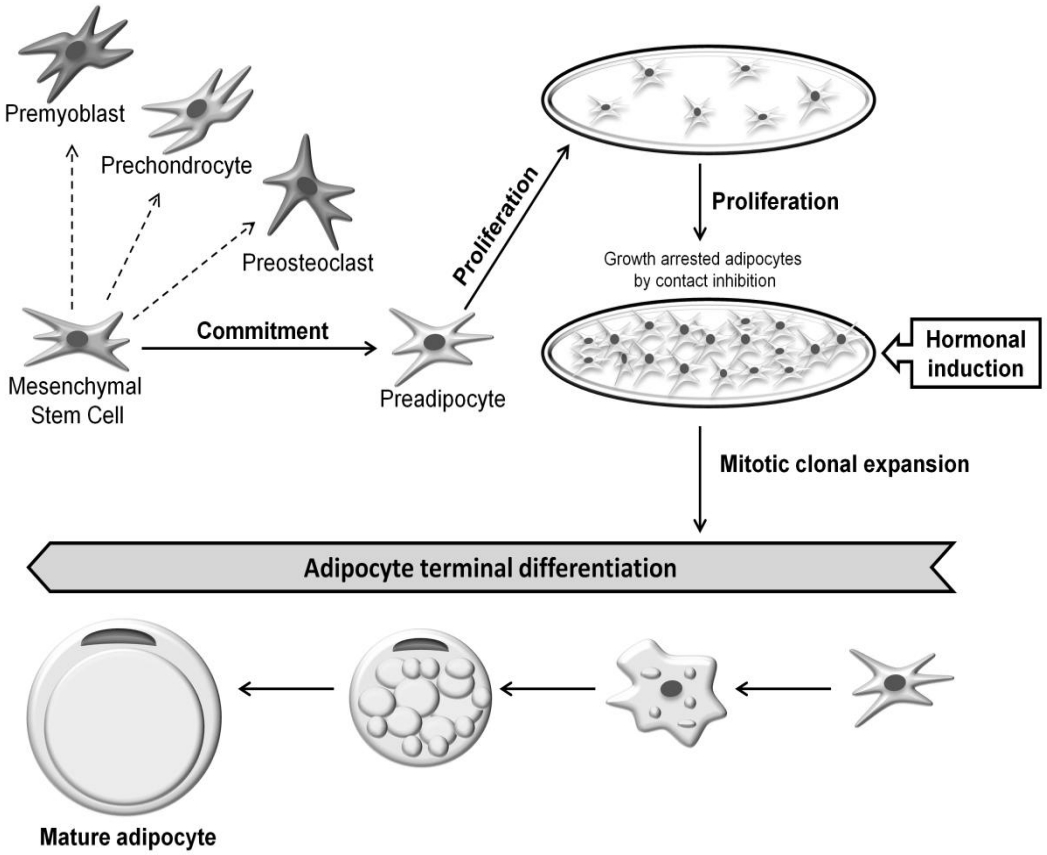

Figure 1.1 Adipocyte commitment and differentiation in cell cultures

MSCs (present in vascular stroma of adipose tissue) are multipotent cells able to commit not only to becoming preadipocytes, but also premyoblasts, prechondrocytes and preosteoblasts (Moreno-Navarrete and Fernández-Real, 2012). After MSCs commit to form preadipocytes they proliferate until growth arrest by contact inhibition. The differentiation program is triggered under an adequate adipogenic stimuli (hormonal induction) leading preadipocytes to turn into mature lipid assimilating adipocytes.

Commitment is the process in which the pluripotent stem cells (PSCs) located in the vascular stroma of adipose tissue respond to signal(s) and go through determination into preadipocytes. The adipose lineage cells originate

from PSCs of mesodermal origin. In the vascular stroma of fat tissue in mammals two types of adipocyte precursors are found: pluripotent fibroblasts (stem cells) and unipotent preadipocytes. Once pluripotent fibroblasts commit to the adipose lineage they can only be induced to form adipocytes, and can not differentiate into other cell types. Bone Morphogenic Proteins (BMP) play an important role in the determination or commitment of the PSCs into preadipocytes (Huang et al., 2009). Both BMP2 and BMP4 were shown to induce commitment of C3H10T1/2 pluripotent stem cells into adipocytes. The treatment with these BMPs followed by exposure to differentiation inducers allows nearly all cells to enter the adipose development pathway, express specific adipocyte markers, and acquire the adipocyte phenotype (Huang et al., 2009). Other factors that may play a role in commitment of stem cells to adipose lineage are cell shape and density. For example, plating human mesenchymal stem cells (hMSC) at low density favored the commitment to osteogenic lineage while high density plating favored the commitment to adipose lineage. Regarding the cell shape, the hMSCs that were allowed to adhere, flatten, and spread underwent osteogenesis, while cells that did not spread and assumed a round shape became adipocytes (McBeath et al., 2004).

Differentiation is the process by which preadipocytes (less specialized cells) turn into mature adipocytes (more specialized cells). In cell culture models, in order to start the differentiation process, preadipocytes reach confluence (cell-cell contact) and become growth arrested (cell cycle stop at G0/G1 boundary) due to cell density inhibition, however it is not the confluence, but the growth arrest itself that is responsible for differentiation of preadipocytes (Gregoire et al.,

1998). After confluence (cell-cell contact), after receiving adequate stimuli, (mitogenic and adipogenic inducers) preadipocytes restart synchronously the cell cycle and undergo mitotic clonal expansion. This step consists of at least one round of cell replication and is a required step for the differentiation of preadipocytes into adipocytes in 3T3-L1 cells (Gregoire et al., 1998). It is suggested that the mitotic clonal expansion is needed to unwind the DNA to allow transcription factor to bind to genes involved in adipocyte differentiation (Moreno-Navarrete and Fernández-Real, 2012). The extensive change in cell shape from a fibroblastic to a spherical shape is considered as the first hallmark of adipogenesis. These changes happen simultaneously with changes in components and levels of extracellular matrix and cytoskeletal components. The modifications that these cells undergo are critical for regulation of adipogenesis since they may promote the gene expression of C/EBPα and/or PPARγ which are fundamental adipogenic transcription factors (Gregoire et al., 1998). PPARγ and C/EBPα play an essential role in the differentiation of adipocytes, since both act synergistically to activate the transcription of several genes that promote the adipocyte phenotype (Lefterova and Lazar, 2009).

1.2.2 Types of adipose tissue

There are two types of adipose tissue in mammals, the white adipose tissue (WAT) and the brown adipose tissue (BAT), which differ morphologically and functionally. The amount of WAT in a non-obese human is approximately 10-15 kg of body weight with this tissue specializing in energy storage in the form of

triacylglycerols, the major energy reserve in mammals. The release of triacylglycerols, which is called lipolysis, provides fuel to other organs. These properties make the WAT an important regulator of energy homeostasis. Besides this, white adipose tissue is also involved in other important processes such as gluconeogenesis and lipoprotein synthesis (Large et al., 2004). On the other hand, brown adipose tissue is very different from white adipose tissue. The amount and localization of lipids disposed in adipocytes also differs between WAT and BAT. White fat cells generally have a major lipid droplet occupying most of the cytoplasm (unilocular) while brown fat cells contain multiple (multilocular) small sized lipid droplets (Hansen and Kristiansen, 2006). Differences in mitochondria amount, vascularization and lipid content lead to color difference between WAT and BAT (Hansen and Kristiansen, 2006). BAT can dissipate energy through adaptive thermogenesis leading to heat production (Hansen and Kristiansen, 2006) which is important to newborns as they lack the ability to shiver. In adult humans the function of brown adipocytes was believed to be insignificant, but recent findings have shown that BAT is activated as a mechanism of heat production during cold exposure. More importantly, BAT was more prevalent in lean individuals in comparison to obese, which makes it an interesting target to treat obesity if molecular strategies can be developed to convert white adipose tissue into energy burning brown adipose tissue in obese individuals (van Marken Lichtenbelt et al., 2009).

1.2.3 Fat cell metabolism

Most of the lipids stored in adipose tissue are triacylglycerols consisting of three molecules of fatty acids and one molecule of glycerol. The synthesis and storage of triacylglycerol in adipose tissue may utilize fatty acids from diet, considered the main source, or from *de novo* lipogenesis (Large et al., 2004). *De novo* lipogenesis consists of the synthesis of fatty acids from substrates that are not lipids, such as carbohydrates and proteins with glucose as the main substrate (Large et al., 2004). The glucose obtained after a meal is primarily metabolized in muscle and adipose tissue under the regulation of hormones such as insulin and catecholamines. Glucose can be directed to *de novo* fatty acid synthesis in the liver and adipocytes, with the fatty acids stored as triacylglycerols in adipocytes. Dietary fatty acids are usually absorbed by the intestine and released into the lymphatic system and then to the blood stream as chylomicrons while the triacylglycerides produced in the liver enter the blood stream as very low density lipoproteins. Both can be hydrolyzed by lipoprotein lipase, and released free fatty acids which can be taken up by adipocyte and stored as triacylglycerols (Large et al., 2004).

Intracellular lipolysis in adipocytes results from the coordinated catabolism of triacylglycerols from lipid droplets which are organelles that regulate storage and hydrolysis of lipids. Triacyglycerols are eventually catabolized to one molecule of glycerol and three molecules of unesterified fatty acids. This is a sequential process involving a number of triglyceride hydrolase enzymes (i.e., hormone sensitive lipase, adipose triglyceride lipase, and

monoglyceride lipase) necessary for adequate hydrolysis of triglycerides, diglycerides, and monoglycerides (Large et al., 2004; Wang et al., 2008b). Other factors can influence lipolysis, such as perilipins and potentially some lipid droplet surface proteins that regulate this process by exposing or protecting the triglycerides in the core of the droplet from the action of lipases (Wang et al., 2008b). Free fatty acids are generally released from adipocytes during fasting periods as an important energy source to various organs, playing an important role in glucose homeostasis. Free fatty acids circulating in the blood stream promote the reduction of glucose uptake by adipocytes and muscles, and also increase the hepatic production of glucose. This strategy saves the carbohydrates for neurons and red blood cells that are dependent on this fuel, while it uses the lipids reserves as an energy source for other tissues (Rosen and Spiegelman, 2006). Lipolysis is under control of endocrine regulation in which insulin acts as an anti-lipolytic factor, while catecholamines promote lipolysis (Wang et al., 2008b). Lipolysis may also vary according to other factors such as species, age, sex, location of fat depots, as well as developmental issues (Wang et al., 2008b).

1.2.4 Adipokines

The number of identified regulatory factors released by adipose tissue has continuously increased over the years and today several adipokines are known to be involved in different biological processes including adiponectin, apelin, chemerin, leptin, omentin, resistin, retinol binding protein 4, tumor necrosis factor-α (TNF-α), vistatin, vaspin, as well as others for which the physiological

function remains to be established (Rosen and Spiegelman, 2006; Galic et al., 2010).

Leptin is the most well studied adipokine and was one of the first signaling molecules detected in adipocytes (Galic et al., 2010). It represses food intake and promotes energy expenditure by targeting hypothalamic cell populations, inducing anorexigenic factors and inhibiting orexigenic neuropeptides (Rosen and Spiegelman, 2006; Lago et al., 2009). Leptin level in circulating serum is directly correlated with the total fat mass of an individual, is positively correlated to insulin and negatively correlated to glucocorticoid levels. Leptin synthesis is mainly controlled by ingestion of food and hormones involved in the regulation of food intake, but its production can also depend on factors such as energy status and sex (Lago et al., 2009).

Adiponectin is a hormone produced by WAT, and unlike other adipokines, concentration in the plasma is negatively correlated with fat mass. Therefore, low levels of adiponectin are associated with obesity while higher levels are associated with weight loss (Rosen and Spiegelman, 2006; Galic et al., 2010). Adiponectin stimulates fatty acid oxidation and glucose uptake in muscle and adipose tissue (Galic et al., 2010) while inhibiting gluconeogenesis in the liver, and it has also been implicated to improve the cardiovascular health (Lago et al., 2009).

Resistin is a peptide produced by adipocytes in mice, but in humans it is produced by mononuclear cells, such as macrophages associated with adipose tissue and macrophages from other organic locations. Resistin has been reported to impact glucose metabolism by reducing the action of insulin in liver and

muscle, and it seems to be involved in a series of biological processes contributing not only to insulin resistance but it may also be involved in inflammatory, endocrine and tumor diseases (Filková et al., 2009).

Omentin is a depot specific adipokine that is produced by stromal vascular cells located in visceral fat depots. It has effects on glucose uptake and works as an insulin sensitizer. It alters the function of the gastro-intestinal barrier that is regulated by insulin and glucose levels (Rosen and Spiegelman, 2006; Poulos et al., 2010b).

TNF-α was the first adipokine identified to be involved in a link between obesity, inflammation and diabetes. Studies have shown that TNF-α influences energy homeostasis, inhibits insulin signaling in adipose tissue and liver and also influences adipose tissue function and expansion. The induction of insulin resistance promoted by TNF-α happens by suppression of proteins involved in insulin dependent uptake of glucose and by repression of PPARγ expression (Galic et al., 2010; Poulos et al., 2010b).

1.3 EXPERIMENTAL MODELS TO STUDY ADIPOGENESIS

1.3.1 *In vitro* models

In vitro models have been very useful for the study of adipogenesis. There are several types of cells with which to study adipogenesis *in vitro* such as preadipocytes cell lines, mature adipocyte-derived dedifferentiated fat cells, mesenchymal stem cells, and adipose derived stem cells.

Preadipocyte cells lines comprise cells that are already committed to adipose lineage. 3T3-L1 and 3T3-F442A cell lines are most frequently used and

were isolated from the Swiss 3T3 cell line, which was derived from 17–19-day-old Swiss 3T3 mouse embryos. It is considered that 3T3-F442A cells are more advanced in the process of commitment than 3T3-L1 cells (Gregoire et al., 1998). These clonal cell lines are very homogeneous in terms of cellular population and are at the same stage of differentiation. These are important features as cells respond homogeneously to treatment. Another advantage is that they can be passaged indefinitely; thereby providing a constant source of cells for study. These cells resemble fibroblasts during the proliferation stage, but after induction they turn into spherical cells filled with lipids displaying morphological and biochemical characteristics similar to adipocytes *in vivo* (Moreno-Navarrete and Fernández-Real, 2012).

Mature adipocyte-derived dedifferentiated fat cells are a relatively new cell model for the study of adipogenesis. These cells originate from mature adipocytes that undergo dedifferentiation, which means that they return from a differentiated state (mature adipocyte) to an immature state resembling a fibroblast. This process happens when mature adipocytes are cultured in a proliferation media (e.g. Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum). During this process the cells lose their lipid droplets, spread and acquire a fribroblastic shape (Matsumoto et al., 2008). Dedifferentiated fat cells are obtained using a culture technique called "ceiling culture". Dedifferentiated fat cells are capable of proliferating and differentiating into multiple cell types, such as adipose, osteogenic and chondrogenic lineages, a

characteristic similar to that of adult stem cells (Matsumoto et al., 2008; Moreno-Navarrete and Fernández-Real, 2012).

Mesenchymal stem cells are also able to differentiate into different cell types, including muscle, bone, cartilage, and adipose tissue. C3H10T1/2 cells are a well-known mesenchymal stem cell line established from CH3 mouse embryos. Unlike 3T3-L1 cells, C3H10T1/2 cells do not promptly differentiate upon exposure to adipogenic inducers, as they require bone morphogenic protein 4 (BMP-4) to be first induced for commitment to the adipose lineage (Armani et al., 2010).

Adipose stem cells are derived from the stromal vascular (SV) fraction of adipose tissue. This fraction corresponds to the pellet of cells remaining after enzymatic digestion to separate the cells followed by the centrifugation of adipose tissue (Poulos et al., 2010a). It consists of several types of cells including, preadipocytes, endothelial cells, fibroblasts, monocytes, and red blood cells. Considering this cell diversity, in order to use SV cells for adipogenesis studies, purification of preadipocytes is required. Preadipocytes isolated from the SV fraction are primary cells and are likely to be more representative of *in vivo* conditions than other cells models. These cells can not be passaged indefinitely, however, and are influenced by the location of the fat depot that they are isolated from and the age of the donor (Armani et al., 2010).

1.3.2 *In vivo* models

In vitro models are great tools to study adipogenesis, however the results obtained from cell cultures may not accurately represent the conditions found in adipose tissue. Adipose tissue consists not only of adipocytes, but several other cellular types including preadipocytes, endothelial cells, fibroblasts, monocytes, and red blood cells (Armani et al., 2010). Since traditional cell lines represent only preadipocytes, these models lack the complexity of adipose tissue which is composed of several cellular types. Other approaches such as the culture of tissue explants or tissue slices may be a better representation of *in vivo* adipose tissue, as the cells and connective tissue are intact. However, deprivation of oxygen, nutrients, and growth factors can cause apoptosis. Therefore, after a few hours there will be some extent of damage to the tissue which makes explants unsuitable for long term experimentation (Novakofski, 2004).

Only *in vivo* models are able to fully represent the complex anatomy and physiology of adipose tissue. In vivo models lend themselves to more readily phenotypic traits such as age of the individual, impact of fat depot, diet and others (Speakman et al., 2007). However, studying adipogenesis using *in vivo* models is challenging, because there is less control over experimental conditions and there is more individual variation as compared to *in vitro* models.

Several species have been used to elucidate metabolism in adipose tissue *in vivo* under different physiological and pathological conditions. The main use of *in vivo* models in the context of adipose tissue is to study obesity. There are several rodent models that are used to study adipogenesis, especially mice, for

example the ob/ob and db/db mouse models, Lethal Yellow Mutant mouse (A^y), New Zealand Obese (NZO) mouse, Tsumura Suzuki Obese Diabetes (TSOD) mouse, M16 mouse, and Kuo Kondo (KK) Mouse. Rat obesity models have also been established such as Zucker Fatty Rat (ZFR), Wistar Fatty Rat and Otsuka Long Evans Tokushima Fatty (OLETF) Rat. These *in vivo* models have been important to advance the knowledge of the pathophysiology of obesity in humans and to further understand the functions of adipose tissue (Kanasaki and Koya, 2011).

Farm animals have also been used as *in vivo* models to further understand the regulation of adipose tissue as fat is an important aspect in meat quality and animal productivity (Hausman et al., 2009; Dodson et al., 2010b). The use of *in vivo* farm animal models to study adipose tissue function aims to understand how adipogenesis is regulated and to develop methods/strategies to manipulate adipose tissue to improve meat quality and animal productivity.

1.4 ADIPOGENESIS REGULATORY MECHANISMS

1.4.1 Transcriptional regulation [2]

1.4.1.1 Transcription factor biology

Transcription is the first step in gene expression, a process in which the DNA of a specific gene is transcribed into RNA. The regulation of transcription is dependent on proteins known as transcription factors which are capable of selectively binding to specific DNA sequences within gene regulatory regions to

[2] Part of the transcriptional regulation section is part of a paper published: Romao, J.M; Jin, W.; Dodson, M.V.; Hausman, G.J.; Moore, S.S.; Guan, L.L. 2011 MicroRNA in mammalian adipogenesis. Experimental Biology and Medicine, 236:997-1004.

control transcription. Transcription factor binding is not the only modulator of gene transcription as other regulatory factors and RNA polymerase also play a role (Latchman, 1997). In most cases, this binding leads to activation of transcription, but there are also cases in which transcription factors promote the repression of transcription (Latchman, 2001). Transcription factors have a modular structure in which certain regions are able to bind to specific DNA sequences while others are capable of producing stimulatory or inhibitory effects on DNA transcription (Latchman, 1997). The human genome contains approximately 20,000 to 25,000 genes and each of them is likely to have a particular spatial and temporal expression pattern. However, the number of DNA binding transcription factors is only a fraction of that total, approximately 1850, which would provide far less patterns of expression than the number of genes. This discrepancy can be overcome by the fact that promoter regions of the genes may have multiple regulatory elements allowing the combinatorial action of different transcription factors, leading to highly specific regulation of gene expression (Maston et al., 2006).

1.4.1.2 Adipogenesis transcriptional cascade

A cascade of transcriptional factors is activated in preadipocytes after they are treated with adipogenic inducers, promoting a temporally regulated set of gene expression events that eventually lead to the adipocyte phenotype (Otto and Lane, 2005; Rosen and MacDougald, 2006). This transition involves a shift in the expression of genes encoding certain transcription factors that dictate the

preadipocyte state to genes that promote the adipocyte phenotype (Rosen et al., 2000). The PPARγ and C/EBP family transcription factors are considered as the master regulators of adipogenesis (Figure 1.2), while other transcription factors can also have a positive or negative regulatory effect on adipogenesis (Lefterova and Lazar, 2009). The transcriptional regulation cascade leads to the acquisition of the adipocyte phenotype which is associated with changes in the expression of more than 2000 genes (Guo and Liao, 2000).

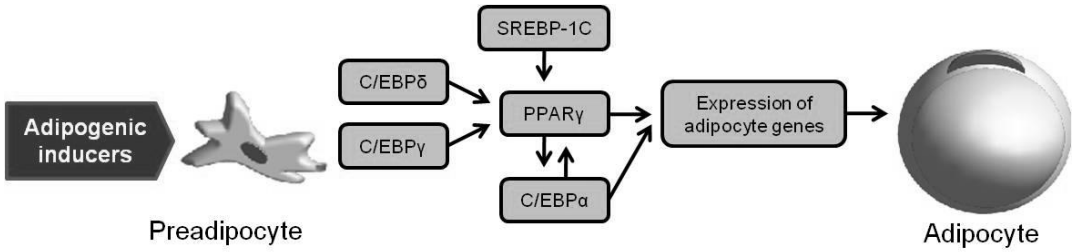

Figure 1.2 Transcriptional cascade of the main pro-adipogenic genes

The transcription factors C/EBPδ and C/EBPγ are the first detected, followed by PPARγ which activates C/EBPα which has a positive feedback on PPARγ, and both maintain the differentiation program, promoting the expression of the terminal differentiation genes that lead to the adipocyte phenotype. Other factors may act as pro-adipogenic or anti-adipogenic in this network, such as SREBP-1c which can also play a role in the activation of PPARγ.

1.4.1.3 Transcription factors in adipogenesis

PPARγ is a member of the nuclear receptor superfamily and is considered to be the most important regulator of adipogenesis (Rosen and MacDougald, 2006). The *PPARγ* gene gives origin to two messenger RNAs that encode two protein isoforms: PPARγ1 and PPARγ2 (Zhu et al., 1995). The retroviral

expression of *PPARγ2* promotes the differentiation of cultured fibroblasts (Tontonoz et al., 1994b) and no other factor is reported to promote fat cell development without the participation of PPARγ (Rosen and MacDougald, 2006). PPARγ can activate several genes including lipoprotein lipase (Schoonjans et al., 1996), acyl-coenzyme A synthetase (Schoonjans et al., 1995), intracellular lipid binding protein (aP2) (Tontonoz et al., 1994a), and phosphoenolpyruvate carboxykinase (Tontonoz et al., 1994a), all of which are responsible for different aspects of fat metabolism.

Because nuclear localization of PPARγ protein follows PPARγ activation by PPARγ ligands, immunolocalization studies are worthy means of studying the functionality of PPARγ (Fernyhough et al., 2007). Immunocytochemistry for PPARγ, C/EBPα and C/EBPα proteins during preadipocyte differentiation coupled with lipid staining in fetal stromal vascular (SV) cell cultures showed that nuclear PPARγ protein was developmentally linked with lipid accretion in differentiating preadipocytes. Therefore, regulation of adipogenesis in fetal SV cultures may ultimately depend, in part, on PPARγ protein expression and nuclear localization in cells with C/EBPα reactive nuclei. Ultimately, this is consistent with the C/EBPα and PPARγ reciprocally regulation of one another to ensure the maintenance of the adipocyte differentiated state.

Several C/EBPs transcription factors participate in adipogenesis. Various members of C/EBP family are expressed in adipocytes: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and *C/EBPζ* (Rosen and MacDougald, 2006). The expression of *C/EBPβ* and *C/EBPδ* precede the expression of *C/EBPα* in the transcriptional

cascade of adipogenesis. Both C/EBPβ and C/EBPδ induce the expression of *PPARγ* (Wu et al., 1996). Once *PPARγ* and *C/EBPα* are expressed in the cell, they synergistically regulate each other's expression, committing the cells to adipocyte differentiation (Shao and Lazar, 1997). C/EBPα induces many adipocyte genes and has an important impact on adipogenesis (Rosen and MacDougald, 2006). In an experiment using transgenic mice that expressed *C/EBPα* in the liver but not in other tissues, it was observed that these individuals developed almost no white adipose tissue, except within the mammary glands (Linhart et al., 2001). On the other hand, C/EBPζ, can act as an anti adipogenic factor that can form heterodimers with other C/EBPs, not allowing them to bind to DNA that leads to inhibition of adipogenesis (Darlington et al., 1998).

Krüppel-like factors (KLFs) are members of a large group of zinc-finger transcription factors that also play a role in adipogenesis. They are known to participate in cell differentiation and development in mammals. KLF can act as negative regulators (*KLF2, KLF 3, KLF 7*) or positive regulators (*KLF 4, KLF 5, KLF 6, KLF 15*) of adipogenesis, by different mechanisms including crosstalk with *PPARγ* and C/EBPs (Brey et al., 2009; Lefterova and Lazar, 2009). Sterol regulatory element binding proteins (SREBPs) are a helix-loop-helix transcription factors expressed in adipocytes. Three members of this family have been identified: SREBP-1a, SREBP-1c and SREBP-2. This transcription factor regulates the transcription of genes involved in lipid metabolism. It was observed that its overexpression in adipocyte cells increased adipogenic activity of PPARγ,

while the ectopic expression of a dominant negative SREBP-1c decreased adipocyte differentiation (Kim and Spiegelman, 1996; White and Stephens, 2010).

Other than the transcription factors discussed above, there are many other factors that seem to play a role in the regulation of adipogenesis, such as: AP-1 transcription factors, STAT proteins, among others (Rosen and MacDougald, 2006; Lefterova and Lazar, 2009; White and Stephens, 2010).

1.4.1.4 Transcriptomic and proteomic studies

Most studies aiming to understand the molecular regulation of adipogenesis rely on measuring the gene expression at the mRNA level. Several technologies are available and have been used for measuring gene expression in adipose tissue/cells including qRT-PCR, quantitative PCR arrays, microarrays and RNA-Seq (Basu et al., 2012). Some of these techniques such as microarrays and RNA-Seq are highly advantageous as they are high-throughput technologies which allow the measurement of thousands of gene simultaneously. On the contrary, proteomic studies have not yet achieved the depth of analysis due to the complex nature of proteins, however important progress in this area has been made lately (Renes and Mariman, 2013).

Transcriptomic profiling has been performed using RNA-Seq and microarrays in several species in the context of adipose tissue and adipogenesis. Many of them have focused on the molecular mechanisms underlying adipogenesis and obesity, but there are also studies on companion animals and adiposity traits in farm animals.

High throughput studies have showed that adipogenesis requires a complex molecular regulation, as a total of 540 genes were differentially expressed (>2 fold) in a microarray study that induced bone marrow-derived human mesenchymal stem cells to differentiate into adipocytes (Hung et al., 2004). Changes in gene expression have also been detected by microarray experiments when different adipose depots are compared in lean and obese individuals. A total of 545 genes were up-regulated in omental adipose tissue and 47 in subcutaneous adipose tissue in lean individuals, revealing differences in molecular regulation among fat depots. However, a different expression pattern was observed in obese individuals as 723 genes were down-regulated in omental adipose tissue and 27 genes in subcutaneous adipose tissue (Hurtado del Pozo et al., 2011). Dysfunction in adipose tissue may also lead to changes in the molecular regulation of other tissues. It was observed in a study using microarray that 1,951 genes were differentially expressed in the blood of individuals with obesity in comparison to lean individuals (Das and Rao, 2007).

In farm animals the use of high throughput techniques to measures gene expression in adipose tissue has gained attention as understanding the molecular regulation of adipose tissue is an important step to develop strategies to improve meat quality. For example, in pigs a deep sequencing study identified 1,596 genes differentially expressed between subcutaneous adipose tissue of pigs with a lean or obese phenotype from different breeds (Li et al., 2012). In beef cattle, studies using next generation sequencing technology (Jin et al., 2012) and microarray approaches (Taniguchi et al., 2008a) identified hundreds of genes (650 from Jin et

al. and 360 from Taniguchi et al.) differentially expressed (DE) in the subcutaneous tissues between individuals with high or low backfat thickness.

The previous studies and several others using high throughput methods to analyze the transcriptomic profiles of adipose tissue in humans, rodents, companion animals and farm animals are advancing our understanding on adipose tissue molecular regulation in order to develop therapies to control adipose tissue dysfunctions such as obesity, but also to improve the quality of meat and productivity of livestock species.

Proteins are a great resource to understand biology as they are the main actors in the cellular context and perform most of the functions dictated by the genome. These molecules are the final product of gene expression and estimates indicate that there are up to one million different types of proteins in humans, which represents a number incredibly higher than the amount of protein coding genes (20,300 genes) (Legrain et al., 2011). This great diversity is due to several factors including alternative splicing, and a variety of post-translational modifications that affect how proteins function and their occurrence may depend on diverse conditions in the cell or organism (e.g. time, location, physiological state and diseases) (Legrain et al., 2011). Proteins can also be influenced by post-transcriptional regulatory mechanisms such as RNAi that can regulate the amount of proteins in a cell through transcriptional repression (Baek et al., 2008).

Proteome profiling studies have also been performed to elucidate the biology of adipose tissue, however they are less abundant and the technologies to profile protein expression is not as powerful as transcriptomic techniques when it

comes to the number of proteins that can be evaluated simultaneously. This limitation is due to the complex nature of proteins and the lack of a technique to measure/identify all proteins (Renes and Mariman, 2013). Nowadays, three main strategies have been applied to proteome research: gel electrophoresis with 1 or 2 dimensions (2-DE) followed by mass spectrometry; liquid chromatography followed by tandem mass spectrometry (LC-MSMS); and antibody arrays (Renes and Mariman, 2013).

Studies aiming to understand the changes in protein expression in adipocytes started in 1979 with the use of 2-DE which showed differences in the expression of several proteins during 3T3-L1 adipocyte differentiation (Sidhu, 1979). Since then several experiments were designed to investigate several aspects of adipocyte and adipose tissue biology including adipogenesis in different types of adipose cells with several studies focusing on different cellular compartments or protein groups. In one of these studies the researchers were able to identify 3,287 proteins in 3T3-L1 cells using sub-cellular fractioning (nuclei, mitochondria, membrane, and cytosol) combined with a high sensitivity protein identification technology (Adachi et al., 2007). Adipose tissues (white and/or brown adipose tissue) have also been subjected to proteomic analysis from several animal species (Salgado-Somoza et al., 2010; Zhao et al., 2010). Other studies have undertaken proteomic profiling of the secretome of adipocytes (Renes and Mariman, 2013). Proteomic research studying the adipose tissue secretome have confirmed the importance of adipose tissue as an endocrine organ by secreting adipokines that may affect neighbor cells in adipose tissue (paracrine) or enter the

blood stream and affect other organs (endocrine). More than 600 adipokines have been identified to be secreted by adipocyte models through 2-DE and LC-MS/MS (Lehr et al., 2012).

Proteome research is able to provide evidence of the molecular regulation of adipocyte/adipose tissue biology that is closer to the final cellular/organism phenotype when compared to transcriptomic research. It is expected that with the further advancements in mass spectrometry and bioinformatics, proteomic analysis will develop greater depth which will further advance our understanding on adipose tissue biology (Renes and Mariman, 2013).

1.4.2 Post-transcriptional regulation

1.4.2.1 MicroRNA biology

MicroRNAs (miRNAs) are small single stranded RNAs molecules that do not encode proteins, ranging from 21 to 25 nucleotides in length (Wahid et al., 2010). These molecules are transcribed from DNA, and reports show that microRNA coding sequences can be located either in both intronic as well as intergenic regions. It is also relevant to mention that most of the miRNA genes are located in close proximity to other miRNA genes (Wahid et al., 2010). MicroRNA genes are generally transcribed by polymerase II (Pol II), but can also be transcribed by polymerase III (Pol III). The product of transcription by Pol II or Pol III is a primary miRNA, also known as pri-miRNA (see figure 1.3) that can be several kilobases, morphologically consisting of a terminal loop structure, stem

loop, double stranded RNA section and two single stranded RNA sections (Wahid et al., 2010).

Processing of miRNAs in animals differs from that in plants, as in the latter miRNAs are completely processed in the nucleus (Jones-Rhoades et al., 2006). In animals, miRNA are processed in the nucleus as well as in the cell cytoplasm. After transcription, the primary miRNA is processed by enzymes: Drosha and DiGeorge syndrome critical region in gene 8 (DGCR8), which cleaves the primary miRNA on the single stranded RNA sections, originating the precursor miRNA (pre-miRNA) which is approximately 70 nucleotides of length (Lee et al., 2003; Wahid et al., 2010). Precursor miRNAs are further processed in the cytoplasm to form mature double stranded miRNAs. At this point they are exported out of the nucleus via nuclear pore complexes, with the transportation mediated by a RanGTP-dependent nuclear transport receptor exportin-5 (EXP5) (Lund et al., 2004).

In the cytoplasm, precursor miRNA is processed by an endonuclease cytoplasmic RNase III enzyme Dicer to form the mature microRNA duplex which is approximately 22 base pairs in length (Wahid et al., 2010). The mature miRNA duplex is loaded into an Ago family protein complex in which one of the strands of miRNA duplex is degraded (miRNA*) while the other remains in the in the Ago family protein complex, also known as microRNA induced silencing complex (miRISC). Once the Ago family protein complex is loaded with the mature miRNA, the complex is active and the miRNA can guide the complex to its target messenger RNA to promote silencing by mRNA degradation or

translation repression (Kim et al., 2009). Figure 1.3 summarizes the several steps of miRNA synthesis and action.

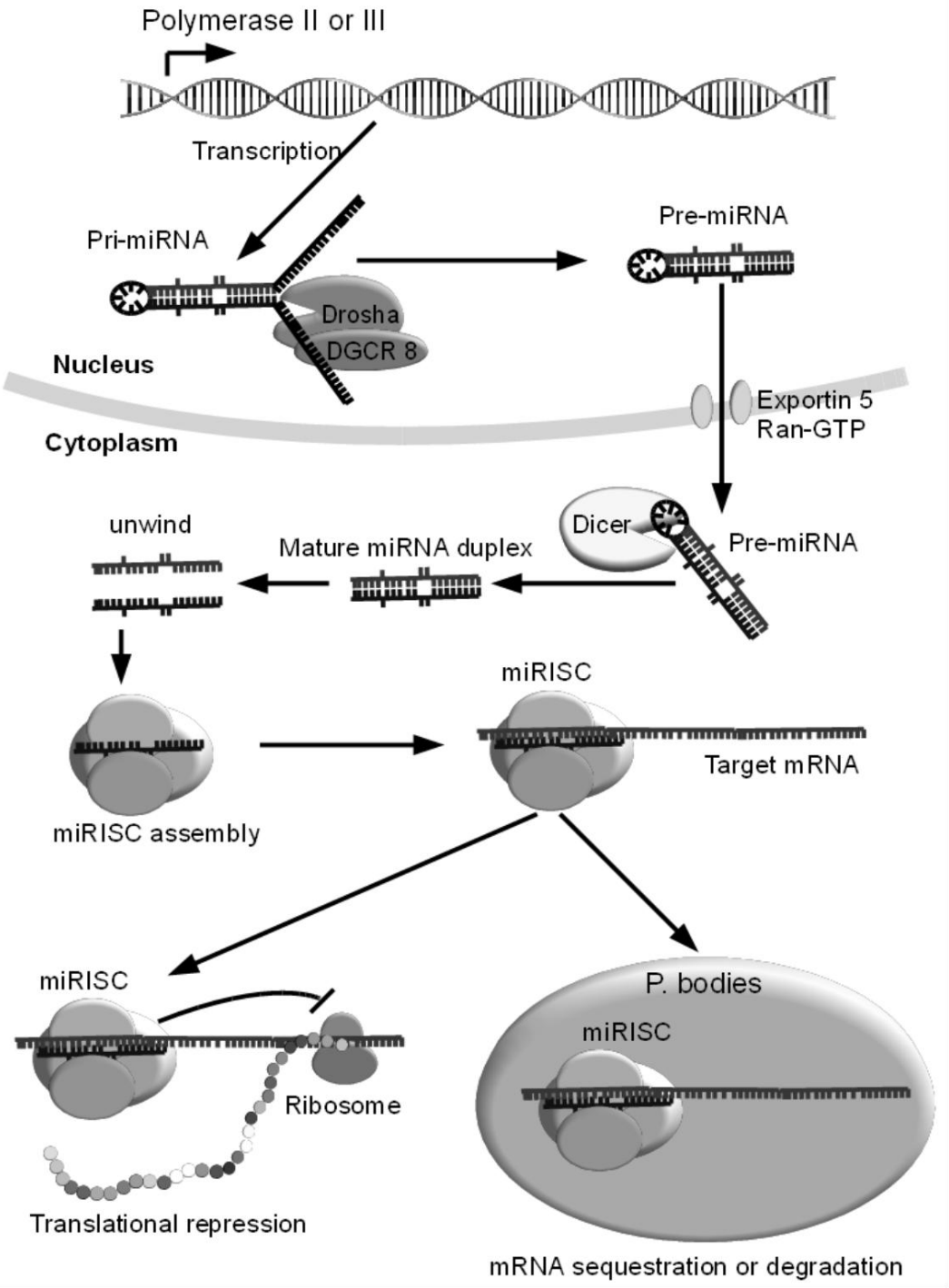

Figure 1.3 Biogenesis and functions of miRNAs

MicroRNAs target messenger RNAs based on the complementarity of their sequences, and mRNA generally are targeted in the 3'UTR. The effect of the activity of a miRISC on a certain mRNA represents a down regulation of the gene expression of the gene that originated the same mRNA. In this manner, regulation of gene expression takes place at a post-transcriptional level and can be promoted by two different ways: translation repression or mRNA degradation (Wahid et al., 2010).

The level of complementarity between miRNA and mRNA is believed to be a major factor in determining the fate of mRNA. A high complementarity may lead to a down regulation of gene expression by degradation of the target mRNA, while a central mismatch between miRNA and mRNA sequences favors translational repression (Wahid et al., 2010). Studies report that translational repression may occur by various methods, such as miRISC binding to target mRNA, repressing the translation at the cap recognition stage, or miRISC binding to mRNA obstructing the translation at the 60s recruitment stage. Another strategy may be that miRISC can prevent the mRNA from circularizing and finally the binding of miRISC to mRNA may promote the separation of ribosomes from mRNA (Wahid et al., 2010).

Additionally, miRNAs are also suggested to perform translational repression by sequestering target mRNAs to processing bodies (P. bodies) in the cell, an area in the cell that is devoid of translational machinery (Valencia-Sanchez et al., 2006). Degradation of mRNA by miRISC is favored by high complementarity of miRNA and mRNA. This process can happen by a guided

cleavage of the target mRNA, but recent studies also show that other processes may also be involved such as decapping, deadenylation, and exonucleolytic digestion of messenger RNA (Behm-Ansmant et al., 2006; Wahid et al., 2010).

By regulating the expression of target genes, microRNAs participate in the regulation of many biological processes in animals, varying from physiological processes such as cell differentiation and proliferation, immune defense to pathological conditions (Williams, 2008).

1.4.2.2 MicroRNAs in adipogenesis [3]

miRNAs have been reported to regulate several biological processes from embryonic development to diseases. It is estimated that over 60% of messenger RNAs are conserved targets of microRNAs (Friedman et al., 2009). Several studies have shown that miRNAs play an important role in adipogenesis (Romao et al., 2011). miRNAs were first shown to regulate adipogenesis in *Drosophila* (Xu et al., 2003). In 2004, miR-143 was the first miRNA found to be involved in mammalian adipogenesis and since then a number of miRNA have been reported to impact adipogenesis, as shown in Table 1.1. To date, there have been hundreds of miRNAs detected in adipose tissue of different mammalian species (Romao et al., 2011). This section explores the roles of miRNAs involved in the commitment to the adipose lineage, the mechanism that miRNAs employ to enhance or inhibit adipocyte differentiation and how miRNAs are regulated in adipose tissue.

[3] The MicroRNA in adipogenesis section is part of a book chapter accepted for publication: Romao, J.M. & Guan, LL. 2013, Adipogenesis and Obesity. In: MicroRNAs in Regenerative Medicine. Dr. Sen, C.K. (ed). ELSEVIER.

Table 1.1 miRNAs involved in adipogenesis

miRNA	Targets	Function	Species	Cell culture model
miR-let-7	HMGA2	Anti-adipogenic	Mice	3T3-L1
miR-15a	DLK1	Pro-adipogenic	Mice	3T3-L1
miR-17-92	Rb2/p130	Pro-adipogenic	Mice	3T3-L1
miR-21	TGFBR2	Pro-adipogenic	Human	hASCs
miR-27a/b	PPARγ	Anti-adipogenic	Human, mice	3T3-L1, OP9, hMADs
miR-30a/d	RUNX2	Pro-adipogenic	Human	hMADs
miR-30c	PAI1, ACVR1	Pro-adipogenic	Human	hASCs
miR-103	-	Pro-adipogenic	Mice	3T3-L1
miR-130	PPARγ	Anti-adipogenic	Human	Primary human preadipocytes
miR-138	EID1	Anti-adipogenic	Human	Adipose mesenchymal stem cells
miR-141	-	Pro-adipogenic	Mice	ST2
miR-143	ERK5	Pro-adipogenic	Human	Primary human preadipocytes
miR-155, miR-221, miR-222	-	Anti-adipogenic	Human	Human mesenchymal stromal cells
miR-199a	SMAD1	Pro-adipogenic	Mice	C3H10T1/2, ATDC5
miR-200a/b/c	-	Pro-adipogenic	Mice	ST2
miR-204, miR-211	RUNX2	Pro-adipogenic	Mice	C3H10T1/2, bone marrow stromal Cells
miR-210	TCF712	Pro-adipogenic	Mice	3T3-L1
miR-369-5p	FABP4	Anti-adipogenic	Human	Human mesenchymal stromal cells
miR-371	-	Pro-adipogenic	Human	Human mesenchymal stromal cells
miR-375	-	Pro-adipogenic	Mice	3T3-L1
miR-378	-	Pro-adipogenic	Mice	ST2, 3T3-L1
miR-429	-	Pro-adipogenic	Mice	ST2
miR-448	KLF5	Anti-adipogenic	Mice	3T3-L1
miR-519	PPARα	Pro-adipogenic	Human	Primary human visceral preadipocytes

1.4.2.2.1 MicroRNAs in adipocyte commitment

Multipotent stem cells from adipose tissue can commit to form adipocytes or generate other lineages of tissues such as bones, cartilage and muscle under the appropriate stimuli. Therefore, a specific molecular guidance directing precursor cells to commit to the adipose lineage must occur in order to form adipocytes. In this context miRNAs seem to play an important regulatory role. For example, mesenchymal stem cells (MSC) induced to differentiate into adipocytes express miR-204. When miR-204 was overexpressed, osteogenesis was inhibited while adipogenesis was promoted. On the other hand when miR-204 expression was inhibited, osteogenesis was increased and adipogenesis impaired. This miRNA targets the runt-related transcription factor 2 (RUNX2), which is a transcription factor involved in osteoblastic differentiation (Alexander et al., 2011). miR-30a/d can also target RUNX2 and has been shown to induce adipogenesis when overexpressed (Hilton et al., 2012). Similarly, other studies reported miRNAs that could impact the fate of stem cells from adipose tissue with direct impact on adipogenesis. The wingless-related MMTV integration site signaling pathway (WNT) can inhibit the commitment into adipose lineage, as observed in MSCs that had osteogenesis favored instead of adipogenesis when they received ectopic expression of WNT10B, which is part of WNT signaling. miR-141, miR-200a/b/c, and miR-429 inhibited Wnt signaling and when overexpressed they induced adipocyte differentiation in ST2 marrow stromal cells (Kennell et al., 2008). miRNAs impact on adipogenic commitment by targeting Wnt signaling may be even more pronounced as a study found 18 potential miRNAs repressing

Wnt signaling and 29 favoring this pathway in 3T3-L1 cells. One of these miRNAs was further analyzed (miR-210) and confirmed to inhibit Wnt signaling by targeting TCF712, which induced increased adipogenesis in cell culture (Alexander et al., 2011). Other miRNAs favor adipogenic commitment by repressing transforming growth factor-beta (TGF-B) signaling. For example, miR-21 targets transforming growth factor, and beta receptor II (TGFBR2) repressing TGF-B signaling. miR-199a targets SMAD family member 1 (SMAD1) which is a regulated by bone morphogenic protein 2 (BMP2), a protein from the TGB-F signaling (Alexander et al., 2011). It is clear that miRNAs play a role in committing multipotent stem cells to the adipose lineage and they may be one of the factors influencing the number of adipocytes in adipose tissue.

1.4.2.2.2 MicroRNAs in adipocyte differentiation

1.4.2.2.2.1 MicroRNAs that increase adipogenesis

Pro-adipogenic miRNAs are those whose regulatory activity favors adipocyte differentiation. Their regulatory mechanism may impact various pathways and different steps of the differentiation process. The direct regulatory impact of miRNAs can be assessed by measuring the level of repression of their targets. In the context of adipocyte differentiation, miRNA regulation can be evaluated by various parameters such as adipocyte numbers and size, intracellular accumulation of triglycerides, differentiation time, expression of adipogenic transcription factors and expression adipocyte markers. For example, the clonal expansion is a key event during adipogenesis of 3T3-L1 cells and is affected by

miRNA regulation. miR-17-92, a cluster that includes miR-17-5p, miR-17-3p, miR-18, miR-19a, miR-20, miR-19b and miR-92-1, is upregulated at the clonal expansion stage during adipogenesis of 3T3-L1 cells. These miRNAs repress the expression of the tumor suppressor protein Rb2/p130, which is part of the 'p130:p107'switch, an important step in the progression of mitotic clonal expansion. When miR-17-92 cluster is overexpressed, adipocyte differentiation is accelerated and triglyceride accumulation increased (Wang et al., 2008a). miR-30c accelerates adipocyte differentiation in human cells as important adipogenic transcription factors were upregulated (PPARγ and C/EBPα) after miR-30c overexpression in cell culture. Plasminogen activator inhibitor-1(PAI-1) and activin A receptor, type I (ACVR1) are validated targets of this miRNA, which might explain its effect on adipogenesis (Karbiener et al., 2011). Other pro-adipogenic miRNAs with known targets include miR-519, which regulates the transcription factor peroxisome proliferator activated receptor alpha (PPARα), which in turn is involved in fatty acid oxidation. There is also miR-15a, which decreases cell proliferation when overexpressed and induces hypertrophy of murine adipocytes by fine tuning of delta like 1 homolog levels (DLK1) (Hilton et al., 2012).

Some miRNAs have an experimentally validated pro-adipogenic action, but their direct targets are still unknown including miR-143, the first miRNA found to be involved in mammalian adipogenesis which is upregulated during pre-adipocyte differentiation. The inhibition of miR-143 impairs the differentiation process but when overexpressed by transfection, adipocytes accumulate additional

triglycerides in cell culture (Hilton et al., 2012). miR-103 is upregulated during differentiation of murine adipocytes (3T3-L1) and its ectopic overexpression increases differentiation and lipid accumulation, with similar results being observed in porcine adipocytes. However, in human adipocytes, miR-103 expression does not change significantly, and is stable over a range of different conditions (obese vs. lean, fat depot, gender, cell culture …) (Neville et al., 2011). Therefore, the roles miR-103 plays in adipogenesis may not be the same among different mammalian species (Hilton et al., 2012). miR-378 is induced during adipogenesis of ST2 cells and when overexpressed increases the size of triglyceride droplets in the cells, while its knockdown decreases lipid accumulation. This miRNA has also been associated with increased backfat thickness in bovines (Jin et al., 2010). miR-375 is likely to induce adipogenesis by modulating the ERK-PPARγ2-aP2 pathway. miR-371 was shown to increase adipocyte differentiation and might also be involved in epigenetic mechanisms controlling adipogenesis *in vitro* (Bork et al., 2011).

1.4.2.2.2.2 MicroRNAs that inhibit adipogenesis

Adipogenesis is a complex process and the differentiation stage is genetically controlled by transcriptional factors, including the PPARγ, which is fundamental for adipocyte differentiation. This transcriptional factor is the target of miR-27a/b that is down regulated during adipocyte differentiation of murine and human adipocytes cell lines. When overexpressed, miR-27 decreases PPARγ expression and reduces adipogenesis (McGregor and Choi, 2011). miR-130 also targets the 3’

UTR of PPARγ messenger RNA, as well as the coding region. Human primary preadipocytes differentiation *in vitro* is impaired when miR-130 is overexpressed, while adipogenesis is increased when levels of miR-130 are reduced (Lee et al., 2011). miR-let-7 impacts the mitotic clonal expansion step, similar to miR-17-92, however, it performs an inhibitory regulation on this process. miR-let-7 expression is down-regulated from day 0 to day 1 after hormonal induction in 3T3-L1 cell cultures (Sun et al., 2009). It is suggested that this temporal down-regulation event allows the occurrence of mitotic clonal expansion and then its expression increases during adipocyte terminal differentiation. The ectopic expression of pre-let-7a oligonucleotide inhibits mitotic clonal expansion and terminal differentiation. miR-let-7 was suggested to inhibit adipogenesis by decreasing mRNA levels of high-mobility group AT-hook 2 (HMGA2), a transcription factor that regulates proliferation and growth in other processes (Sun et al., 2009). miR-369-5p targets the fatty acid binding protein 4 (FABP4) which is a genetic marker of mature adipocytes. Over expression of miR-369-5p inhibits adipocyte differentiation and also seems to be involved in epigenetic control of adipogenesis(Bork et al., 2011). Other anti-adipogenic miRNAs with known targets include miR-138 which targets adenovirus early region 1-A-like inhibitor of differentiation 1 (EID1) (Yang et al., 2011) and miR-448 which targets kruppel-like factor 5 (KLF5) which is a transcription factor that induces the expression of PPARγ (Kinoshita et al., 2010). Over expression of miR-138 or miR-448 in adipocyte cell cultures reduces adipocyte differentiation (Kinoshita et al., 2010; Yang et al., 2011). miR-155, miR-221 and miR-222 were down-

regulated in human mesenchymal stromal cells when induced to differentiate into adipocytes (Skårn et al., 2012). Over expression of these miRNAs inhibited adipogenesis and repressed induction of the master regulators PPARγ and C/EBPα. However their targets have yet to be experimentally validated (Skårn et al., 2012).

1.4.2.2.2.3 MicroRNA changes during adipocyte differentiation

miRNAs act in concert to regulate biological processes such as cellular differentiation. Expression of some miRNAs does not change during differentiation as they may regulate basic cellular processes, but expression of other miRNAs may change (increase or decrease) to induce or inhibit more specialized cellular processes. The dynamic changes of miRNA expression lead to the inhibition or induction of genes that drive cellular differentiation. For example, adipocytes derived from subcutaneous adipose tissue in humans were isolated and induced to differentiate *in vitro* (Ortega et al., 2010). The expression of a total of 70 miRNAs was altered (±1.2 fold change, $p<0.0001$) when preadipocytes (day 0) were compared to mature adipocytes after differentiation (day 14). The expression of 33 miRNAs increased during differentiation and the most up-regulated miRNAs were miR-378 (+6.6 fold), miR-30c (+5.1 fold), miR-30a (+4.0 fold), miR-30b (+3.1 fold), miR-30e (+3.1 fold), miR-30a-3p (+2.8 fold) and miR-34a (+2.5 fold). While 37 miRNAs had their expression reduced during adipocyte differentiation with the most down-regulated miRNAs being miR-31-3p (-22.6 fold), miR-210 (-24.3 fold), miR-221 (-24.9 fold), miR-424 (-

24.6 fold) and miR-503 (-26.7 fold) (Ortega et al., 2010). Some of the up-regulated miRNAs (miR-378, miR-30a and miR-30c) are known to have a pro-adipogenic action (Gerin et al., 2010; Karbiener et al., 2011; Zaragosi et al., 2011). Similarly, miR-221 has an anti-adipogenic role and was down regulated (Skårn et al., 2012). However, the other miRNAs up and down-regulated do not have an established role in adipogenesis, which shows the need for further investigation to clarify the role of deregulated miRNAs during adipocyte differentiation in humans.

However it is not clear how miRNA expression is regulated in adipose tissue. Some miRNAs have their DNA sequence located between genes (intergenic regions) of the DNA and are transcribed into RNA as independent units while other miRNAs are located inside the host genes, in introns for example. In this case these miRNAs are transcribed simultaneously with the genes that host their sequences. Transcription factors are known to regulate the expression of genes, which may also participate in the regulation of miRNAs by promoting the expression of miRNA genes or the transcription of their host genes. In adipogenesis, PPARγ is the most influential transcription factor since it has thousands of genomic binding sites, indicative of its important regulatory role. A study attempted to find out whether miRNA expression is under the regulation of PPARγ and it identified 22 putative PPARγ target miRNA genes with PPARγ binding sites within ±50 kilobases distance from their transcription start site (John et al., 2012). Some of these miRNAs were located inside protein-coding genes, while others had their own genes. Among these, the study further verified that the

expression of miR-103-1 (inside gene PANK3), miR-148b (inside gene COPZ1), miR-182/96/183 (own miRNA gene), miR-205 (own miRNA gene) and miR-378 (inside gene PPARGC1B) followed that of PPARγ during *in vitro* adipocyte differentiation (John et al., 2012). These findings provided some clarity on how miRNAs might be regulated in adipose tissue and improved our understanding of how the regulatory network of miRNAs and transcription factors control gene expression of adipocytes.

1.4.3 Factors that influence miRNAs in adipose tissue [4]

miRNA expression in adipose tissue changes according to a variety of conditions. Dietary manipulation has already been shown to change expression of protein coding genes (López et al., 2004) and the same is observed with miRNAs (Chartoumpekis et al., 2012). Even early life dietary changes might induce long term miRNA expression changes (Ferland-McCollough et al., 2012). Brown and white adipose tissue express different miRNA profiles (Mori et al., 2012) and changes are also observed among different anatomical locations of fat depots such as visceral, subcutaneous and intramuscular fat (Rantalainen et al., 2011). The influence of dietary changes and adipose depot type are further discussed below.

1.4.3.1 Diet

The expression of protein coding genes in adipose tissue is already known to be impacted by dietary manipulation; however the understanding on how diet

[4] Part of Romao, J.M. & Guan, LL. 2013, Adipogenesis and Obesity. In: MicroRNAs in Regenerative Medicine. Dr. Sen, C.K. (ed). ELSEVIER.

impacts miRNA expression is still scarce. Administration of high-fat diets is the most common strategy used to investigate the effects of diet on miRNA expression as it simulates diet induced obesity models. For example, mice fed a high fat diet for 8 weeks had an increased miR-143 expression in mesenteric adipose tissue and miR-143 levels were highly correlated to the expression of PPARγ, FABP4 and plasma levels of leptin (McGregor and Choi, 2011). miR-27a was down-regulated in mature adipocytes from adipose tissue of mice fed a high fat diet (McGregor and Choi, 2011). Conjugated linoleic acid (CLA) is proposed to promote weight loss and a study reported that CLA supplementation in the diet of mice reduced the expression of miR-143 and miR-107 in retroperitoneal adipose tissue, while it increased the level of miR-222 in this tissue (Parra et al., 2010). These findings reveal that the expression of miRNAs can respond to dietary changes and early life nutrition seems to play a role in miRNA expression.

Researchers fed female rats a low protein diet during pregnancy and lactation which produced offspring with reduced adipose mass and adipocyte size (Ferland-McCollough et al., 2012). Microarray analyses showed that miR-483-3p was the only miRNA up-regulated at 22 days and 3 months of age in the offspring, indicating this change was a sustained programmed event. The over expression of miR-483-3p in 3T3-L1 cell culture inhibited adipogenesis by targeting growth differentiation factor-3 (GDF3) directly, which is required for lipid accumulation during late stages of adipocyte differentiation (Ferland-McCollough et al., 2012). The exact mechanism controlling miR-483-3p programming is still unknown. Interestingly, humans from a similar model of

suboptimal early nutrition (humans with low birth weight) had miR-483-3p expression up-regulated with repressed expression of GDF3, suggesting this is a conserved mechanism with potential long term health consequences (Ferland-McCollough et al., 2012).

1.4.3.2 Adipose depots

Adipose tissue is composed of two types, WAT and BAT. These distinct tissues differ morphologically and functionally. Understanding the differences in miRNA expression regulation between BAT and WAT may provide insight into approaches to control of obesity. In theory, converting WAT into BAT would promote the expenditure of fat reserves into heat, reducing adipose mass and consequently obesity. Evidence shows that miRNA expression in brown adipocytes differs from that in white adipocytes (Walden et al., 2009). For example, expression of miR-143 was lowly in mature brown adipocytes of mice, while it was highly expressed in white adipocytes. miR-455 was upregulated during brown adipocyte differentiation and it was speculated to have a pro-adipogenic role. Interestingly, three miRNAs normally expressed in myocytes (miR-1, miR-133a and miR-206) were not detected in white adipocytes but were expressed in brown adipocytes (Walden et al., 2009). Another study identified miR-196a as a potential controller for converting WAT into BAT (Mori et al., 2012). miR-196a is up regulated during brown adipocyte differentiation and targets the white fat gene homeobox C8 (HOXC8). HOXC8 is down regulated during brown fat adipogenesis and represses the expression of brown fat genes,

including the master regulator of brown adipogenesis. In order to test miR-196a *in vivo,* transgenic miR-196a mice were developed in order to promote forced expression of miR-196a in adipose tissue. The results showed that miR-196a increased the amount of BAT in adipose tissue, increased energy expenditure and promoted resistance to obesity (Mori et al., 2012). These findings open the possibilities of exploring miRNAs as therapeutic tools to control obesity by taking advantage of the differential molecular regulation of BAT and WAT (Mori et al., 2012).

miRNA expression is different not only between WAT and BAT but also among different depots of WAT, such as among visceral, intramuscular and subcutaneous adipose tissues. A high throughput study profiled 1,146 human miRNAs from abdominal and subcutaneous gluteus fat from 70 individuals revealed 136 (12%) miRNAs differentially expressed between the two locations with 61 miRNAs having higher expression in gluteal fat while 75 were higher in abdominal fat (Rantalainen et al., 2011). Distinct miRNA regulation in different fat depots is further supported by a study that found that the correlation between the expression of 95 miRNAs from subcutaneous adipose tissue and that from omental adipose tissue in 50 individuals was not significant ($r = -0.187$, $p=0.07$) (Heneghan et al., 2011). Another study compared miRNA expression between omental and subcutaneous adipose tissue from individuals with normal glucose tolerance and individuals with type 2 diabetes (T2D). These researchers reported that the first group had four miRNAs differentially expressed between fat depots, while twelve miRNAs were differentially expressed in the second group (Klöting

et al., 2009). Different miRNA profiles were reported even in three different locations of the same depot (subcutaneous fat) (Jin et al., 2009), supporting the tissue type effect on miRNA expression. A porcine study using primary adipocytes found 30 miRNAs expressed in a depot-specific manner, of which 24 were only expressed in intramuscular fat while 6 were expressed only in subcutaneous fat (Guo et al., 2012). All these findings from different mammalian species support the notion that different adipose tissues have different miRNA regulation reflecting their diverse physiology.

1.4.4 Systems biology

It was previously thought when the human genome was sequenced in 2001 that only a small fraction of the genome (5-10%) was functional. However, this concept has changed dramatically in recent times. Recently the ENCODE project reported that more than 80% of the components of human genome have at least one biochemical function (Skipper et al., 2012). Besides, evidence shows that up to 90% of the eukaryotic genome can be transcribed, which produces a great amount of non-coding RNAs (Costa, 2010). The latest release of the ENCODE statistics (version 17, February, 2013) shows a total of 57,281 genes in the human genome with 20,330 protein-coding genes, 13,333 long non-coding RNA genes, 9,078 small non-coding RNA genes, and 14,154 pseudogenes (ENCODE, 2013). In this context it is becoming clear that eukaryotic genes can be divided into two big groups: the protein-coding genes and the non-protein coding genes (Costa, 2010).

In order to fully understand how biological processes such as adipogenesis are regulated, it is necessary to investigate how these two groups of transcripts (non-coding and coding) interact with each other and how this affects protein expression. This is a multi-dimensional effort, as it potentially involves the study of the interactions among transcriptome, microRNAome and proteome in a systems biological approach.

Gene expression is the process by which the biological information in the genome is used to synthesize a functional gene product. Protein coding genes are not the only type of gene capable of creating functional products such as proteins. Non-protein coding genes can also carry out this role by producing functional RNAs, for example transporter RNA, ribosomal RNA, small nucleolar RNAs, long non-coding RNAs and microRNAs (Costa, 2010). MicroRNAs and messenger RNAs are two well-known classes of RNAs coded in DNA. Despite having the same structure (formed by nucleotides), the nature of their function is completely different because miRNA are fully functional as an RNA molecule requiring no further processing, while messenger RNA are information molecules which are translated to proteins. Yet these molecules can interact in complex networks (Clarke et al., 2012) and regulate virtually all biological processes in animals.

Gene expression is a highly controlled process with several layers of regulation including epigenetic mechanisms, transcriptional regulation and post-transcriptional regulation. Several studies show that mRNA levels do not correlate well with protein levels. Researchers using samples from liver of mice found an

average correlation of 0.27 between mRNA and protein levels of 396 genes. Only 21% of the genes exhibited a significant positive correlation ($R > 0.46$, $p < 0.001$) between RNA and protein (Ghazalpour et al., 2011). Another study obtained an average correlation of ~0.3 between mRNA and proteins (1,066 genes) using 23 human cells lines (Gry et al., 2009). A high throughput experiment using embryonic cells (NIH 3T3) profiled mRNA and protein levels of 5,000 genes and reported that on average ~40% of the variations in protein levels were directly explained by mRNA levels and most of the variation in protein levels was due to translation rate (Schwanhausser et al., 2011). In light of these findings it becomes clear that post-transcriptional regulatory mechanisms may play an important role in gene expression and consequently protein levels, including miRNAs through transcriptional repression (Baek et al., 2008). Post-translational regulation of proteins is an essential cellular mechanism which is able to change the function and activity of proteins by adding other biochemical functional groups such as small chemical groups, lipids, and even small proteins that can account for more than 200 post-translational modifications (PTMs) (Altelaar et al., 2013). PTMs add an extra level of molecular regulation and allow the number and forms of proteins produced to be much higher than that reflected by the number of genes that code for proteins.

Epigenetics have been known to regulate gene expression by direct DNA modification through cytosine methylation and also by histone modifications which correspond to post-translational modifications of the core histone proteins by different processes such as methylation, acetylation, ubiquitylation, and

phosphorylation, altering the chromatin structure (Capell and Berger, 2013). miRNAs are reported to be part of this system as they target and regulate the expression DNA methyltransferase genes (DNMTs), which encode proteins that establish and maintain the methylation of the fifth carbon of cytosine residues in DNA (Denis et al., 2011) and also histone deacetylases (Sato et al., 2011). Interestingly, DNA methylation and histone modifications not only regulate the expression of protein coding genes, but also of miRNAs (Sato et al., 2011). Therefore, there is a complex interplay between miRNA and epigenetic mechanisms influencing gene expression at transcriptional and post-transcriptional levels. Several biological processes including adipogenesis are likely to be affected, as illustrated by the impact of the early life nutrition of mice and humans epigenetically affecting the expression of miR-483-3p as described previously (section 1.4.3.1 Diet) which impacted the regulation of adipose tissue in adult life (Ferland-McCollough et al., 2012).

Another instance of interplay between molecular regulators in adipogenesis occurs between miRNAs and transcription factors. PPARγ is the main regulator of adipogenesis and evidence shows it does not only regulate the expression of adipogenic genes, but also of several miRNAs (John et al., 2012), while some miRNAs have also been shown to target the messenger RNA of PPARγ gene and control its expression (Kim et al., 2010).

Understanding the regulation of gene expression in adipogenesis is a complex task that cannot be fully accomplished by focusing only on specific categories of molecules. The reason for this is that several different molecules

(e.g. proteins, miRNAs, and mRNAs) and mechanisms (e.g. epigenetics, transcriptional, post-transcriptional regulation and post translational modification) are involved in adipogenesis, and they interact with each other in complex regulatory networks. Furthermore, studying the metabolites (intermediate and products of metabolism usually restrict to small molecules) of adipose tissue is an additional approach to understanding adipose tissue/adipocyte physiology and provides valuable information as to how genomic regulation can impact the phenotypic characteristics of this functional tissue (Roberts et al., 2009). Therefore, a systems biology approach is desirable as it takes into account the complexity of this biological process, being able to provide a more holistic understanding of adipogenesis and adipose tissue physiology.

1.5 ADIPOSE TISSUE IN BEEF CATTLE

1.5.1 Bovine fat

Adipose tissue in beef cattle is distributed in several depots including subcutaneous fat, visceral fat, intra and intermuscular fat. These fat depots have different anatomical locations, morphologies, as well as distinct physiology. For example, a study found in beef steers (~579 kg) a total of 100 kg of fat tissue, of which 16.5 kg was subcutaneous fat, 46.8 kg was intermuscular fat, and 37.3 kg was internal fat (Robelin, 1981). Differences in hypertrophy and hyperplasia of adipose tissue in beef cattle have also been observed as adipose samples with the same mass but from different fat depots possessed different adipocyte sizes and adipocyte numbers (Cianzio et al., 1985). Adipocyte sizes in 17 month old steers

in decreasing order were kidney fat > mesenteric > subcutaneous > intermuscular > intramuscular > brisket fat (Cianzio et al., 1985). Evidence also supports the contention that different fat depots differ in their physiological function as adipose cells derived from different fat depots were found to display distinct proteomic profiles (Rajesh et al., 2010). Subcutaneous and intramuscular fat tissue also had distinct transcriptomic profiles (Bong et al., 2010). Intramuscular fat is considered crucial for the beef industry as this fat depot is responsible for marbling, which is one the main indicators of beef quality. Manipulating (increasing) the amount of intramuscular fat in beef cattle is one of the challenges for the industry in order to provide the consumer with a high quality product (Dodson et al., 2010b). At the same time it is important to control the amount of fat in other fat depots (eg. subcutaneous and visceral fat), as this fat is normally not consumed and excesses have a negative impact on animal productivity (Dodson et al., 2010b).

Adipose tissue in the context of bovine production is not only relevant to beef cattle, but also to dairy cattle. Fat is one of the main components dairy cows secrete to produce milk. It is estimated that a dairy cow producing an average of 30 kg of milk/day during the first weeks of lactation secretes a total of 1.5 Kg of lipids, of which 1 Kg is derived from adipose depots (Barber et al., 1997). Therefore, the lipolysis in adipose tissue plays an important role during the lactation of dairy cows as a major source of fatty acids, especially during negative energy balance when the nutrient demand of the mammary gland may exceed those of the rest of the individual. In non-lactating ruminants adipose tissue assumes the main role in fatty acid synthesis (Nafikov and Beitz, 2007), in

contrast to cows during the lactation period that show reduced lipogenic capabilities in adipose tissue, while mammary gland assume most of the synthesis of lipids (Barber et al., 1997). Therefore, lactating dairy cows represent a different perspective on how adipose tissue is physiologically regulated compared to beef steers.

It is clear that adipose tissue is a complex organ as it is not very uniform consisting of several independent fat depots which have distinct anatomic locations, morphology and physiology. Therefore, strategies to study and manipulate adipose tissue in beef cattle have to take into consideration the complexity of fat depots to succeed.

1.5.2 Meat quality and manipulation of adipose tissue

Meat quality is a measure of how desirable meat is to consumers (Wood et al., 1999) and depends on several factors. For example, physical intrinsic qualities such as tenderness, juiciness, flavor, color, shape, and appearance are very important to meat quality as well as nutritional qualities including the fatty acid profile of fat (Hocquette et al., 2012). The physical intrinsic qualities are influenced by several factors including the genetics of the animal, the type of feed fed and livestock practices. Another aspect that is involved in meat quality is the post-mortem processes involved in the conversion of muscle to meat (Hocquette et al., 2012). Therefore meat quality involves many steps as it may involve aspects along a continuum from animal selection and feeding to meat processing and packaging. Tenderness is considered to be the most important physical

characteristics of meat quality (Wood et al., 1999) and it changes mostly due to muscle fiber characteristics, muscle glycogen content, collagen content and solubility, and also the presence of proteases and their inhibitors during aging (Hocquette et al., 2012). Flavor is also fundamental and it arises in cooked meat as result of reactions between carbohydrates and proteins and also among their sub products. Fat plays an important role in meat quality as its presence is able to increase flavor (Wood et al., 1999). Fat may change according to the composition of different types of fatty acids. The composition of adipose tissue in muscle is reported to affect the firmness of meat as different fatty acids have different melting temperatures (Wood et al., 2008).

Fatty acid composition in beef is also an aspect when it comes to the human health. Adipose tissue from beef is known to be rich in saturated fatty acids which have been negatively linked to cardiovascular diseases (Shingfield et al., 2012). However, beef is also a source of healthy fatty acids such as conjugated linoleic acid (CLA) which can have beneficial health effects (Pariza et al., 2001). Essential fatty acids such as n-3 polyunsaturated fats (eicosapentaenoic acid and docosahexaenoic acid) are normally found in marine fish and studies report their importance to human health as they confer hypotriglyceridemic and anti-inflammatory effects (Siriwardhana et al., 2012). Evidence also supports their potential role as antihypertensive, anticancer, antioxidant, antidepression, antiaging, and antiarthritis agents (Siriwardhana et al., 2012). In this context, studies have been carried out in order to develop strategies to improve beef quality by altering the fatty acid composition to a healthy profile, for example by

increasing the amount of n-3 fatty acids. The main approach to manipulating the fatty acid profile in beef is through dietary means. Studies have reported that the lipid content in the diet and also the ratio between forage and concentrate can determine the products from rumen lipid metabolism and affect fatty acid profiles of ruminant derived foods (meat and milk) (Shingfield et al., 2012). Meat from grain-fed or grass-fed cattle has been reported to have different fatty acid composition with 27 out of 36 fatty acids being affected (Alfaia et al., 2009), including increased omega 3 fatty acids and conjugated linoleic acid (CLA) in grass-fed cattle (Daley et al., 2010). Studies have also focused on other dietary approaches to alter fatty acids composition such as the use of different forage species, different methods of forage conservation, and the use of dietary supplements such as plant oils, oilseeds, fish oil and marine algae (Shingfield et al., 2012). The amount and distribution of fat in the carcass is of great interest for the beef industry as it is another important determinant of meat quality. A sensory panel has shown that each full increase in marbling scores from slightly to slightly abundant lead consumers to display approximately 10% more acceptance to top loin steaks (Platter et al., 2003). In order to take advantage of the impact of marbling on meat quality, efforts have been made to increase the amount of intramuscular fat (marbling) with studies focusing on factors that can impact marbling such as diet (Andrae et al., 2001), management practices (May et al., 1992) and genetic variation (Pitchford et al., 2002).

1.5.3 Transcriptomic and proteomic profiling in bovine adipose tissue

To date, the research on bovine adipocytes/adipose tissue is very limited; therefore there is not a clear understanding on how adipogenesis is genetically regulated in beef cattle. Few research groups including ours have been focusing on studying molecular mechanisms of bovine adipogenesis using both tissue and cell line models. Firstly, studies using preadipocytes reported differential expression of 100 genes between differentiated and control cells including PPARγ, FASN, FABP and others involved in biochemical pathways and cellular/molecular signaling (Taniguchi et al., 2008b). This study also showed that the PPARγ was up-regulated during the early preadipocyte differentiation, while C/EBPα was not differentially expressed during adipocyte differentiation. Together with other evidence (Dodson et al., 2010a), we can conclude that adipogenesis and fat metabolism in bovines differs from that in humans and murines. In addition, other studies using microarray (Taniguchi et al., 2008a) and next generation sequencing technology (Jin et al., 2012) identified hundreds of differentially expressed genes between steers with high or low backfat thickness. From the same tissue samples, when the expression of 86 miRNAs was examined, 42 out of 86 miRNAs were differentially expressed, suggesting that miRNAs may be involved in the regulation of fat traits (backfat thickness) in beef steers. Among the DE miRNAs, miR-378 had the highest fold change (1.99-fold increase), and is located in the intronic region of PPARGC1B (Jin et al., 2010). In addition, even different regions of the same fat depot; abdominal, back and rump subcutaneous fat tissue have different miRNA profiles with a total of 80, 66 and 63 miRNAs

detected in each of them respectively (Jin et al., 2009), a result that suggests that miRNA based regulatory mechanisms in adipogenesis differ among fat depots.

Furthermore, previous work also identified that proteins differed significantly between subcutaneous tissues collected from cattle with high and low levels of backfat (Zhao et al., 2010). In total, 13 proteins were identified, and differential expression of annexin 1 was further verified using western blot (Zhao et al., 2010). However, the annexin 1 mRNA levels did not differ statistically, suggesting that other mechanisms such as post translational and microRNA regulated protein expression may be playing a key role in regulating adipogenesis (Jin, W. personal communication).

Diet is also another factor that influences gene expression in bovine adipose tissue, as different diets were shown to change the expression of genes related to lipid metabolism, including several genes involved in the synthesis of enzymes involved in fatty acid metabolism and lipogenesis such as stearoyl‑CoA desaturase (SCD), fatty acid synthetase (FASN), lipoprotein lipase (LPL) and fatty-acyl elongase (LCE) (Joseph et al., 2010).

Fat depot location also influences the transcriptomic and proteomic profile in bovine adipose tissue. A SAGE (serial analysis of gene expression) experiment analyzed the expression of genes from subcutaneous and intramuscular adipose tissue from Korean cattle revealed that 82 genes were differentially expressed (> 2 fold) between the two fat depots (Bong et al., 2010). Another study using 2-dimensional polyacrylamide gel electrophoresis coupled with sequencing mass spectrometry found that adipose cells isolated from different fat depots (omental

fat, subcutaneous fat and intramuscular fat) presented diverse protein expression profiles indicating that fat depot is a factor that influences protein expression in bovine adipose tissue (Rajesh et al., 2010).

So far, few aspects of bovine adipogenesis have been studied and the understanding on how factor such as diet, depot location and genetic background impact gene expression at both the mRNA and protein level and also the role that miRNAs play in this process is very limited and fragmented. Therefore, further research focusing on these aspects is necessary to elucidate the molecular regulation of bovine adipose tissue under different physiological conditions.

1.6 SUMMARY

Efforts to understand the molecular regulation of adipose tissue have been performed not only to understand the pathophysiology of adipose disorders in humans such as obesity, but also to manipulate adiposity traits in farm animals to improve meat quality and animal productivity. The scientific literature clearly shows the importance of adipose tissue not only as an energy reserve site, but also as a dynamic endocrine organ that contributes to the regulation of energy metabolism. Significant advances have been made in characterizing the molecular regulation of adipocytes through transcriptomic approaches. However, there are several other aspects of adipose biology that are not clear such as the involvement of microRNAs in adipogenesis. Besides, very few studies have attempted to explore the physiology of bovine adipose tissue using the final products of gene expression (proteins).

We hypothesized that external (diet) and internal (fat depot and age) environmental factors affect the regulation of adipose tissue at miRNA and protein levels in beef steers. And also that genomic context features of miRNA genes impact their expression and function. The long term goal of this study is to improve the current understanding of the molecular regulation of bovine adipose tissue under different conditions *in vivo*, allowing the discovery of protein/microRNA markers and leading to the development of strategies to manipulate adiposity in beef cattle. This research represents the preliminary steps required to achieve this long term goal in order to promote improved animal productivity and meat quality. The specific objectives are: to determine the miRNA expression in bovine subcutaneous and visceral adipose tissue in cattle fed different diets (Chapter 2); to elucidate the molecular mechanisms of physiological variations between subcutaneous and visceral fat depots in cattle fed different diets (Chapter 3); to investigate the influence of genomic context of bovine miRNA genes on their expression and function (Chapter 4); and to study the impact of animal growth on the proteome profile of bovine subcutaneous adipose tissue (Chapter 5).

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2 ALTERED MICRORNA EXPRESSION IN BOVINE SUBCUTANEOUS AND VISCERAL ADIPOSE TISSUES FROM CATTLE UNDER DIFFERENT DIETS [5]

2.1 INTRODUCTION

Adipose tissue is dynamic, with its fundamental activity in the regulation of energy balance and its role in endocrine function becoming increasingly evident (Galic et al., 2010; Poulos et al., 2010). Adipogenesis is the process by which preadipocytes differentiate into adipocytes (Large et al., 2004). The extent of adipogenesis is influenced by a number of factors including diet, fat depot, age and breed (Kirkland et al., 2002; Hosooka et al., 2008; Jin et al., 2012). Studies have shown the importance of adipogenic transcription factors (PPARγ, C/EBPs, KLFs and SERBP), which regulate the expression of many adipogenic genes that participate in adipocyte differentiation (Lefterova and Lazar, 2009; White and Stephens, 2010).

A class of gene regulators known as microRNAs (miRNAs) have been discovered to regulate gene expression in many biological processes including embryo development, differentiation, apoptosis, and metabolism in animals (Krützfeldt and Stoffel, 2006; Lee et al., 2006; Song and Tuan, 2006). These molecules are small non-coding RNAs with approximately 22 nucleotides that are able to repress gene expression by binding to messenger RNAs in a sequence-specific manner (He and Hannon, 2004). Such regulatory roles appear to be tissue

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specific as many tissue specific miRNAs have been identified (Lagos-Quintana et al., 2002a; Liang et al., 2007). Recent studies reported that adipogenesis in humans and mice is also regulated by miRNAs, with several miRNAs being reported to have pro or anti-adipogenic roles (Xie et al., 2009b; Romao et al., 2011) through repression of various genes, including transcriptional factors such as PPARγ, PPARα and KLF5 (Lin et al., 2009; Kim et al., 2010; Kinoshita et al., 2010; Martinelli et al., 2010). miRNAs have also been shown to be differentially expressed in bovine adipose tissue with the expression of miR-378 expression varying with thickness of subcutaneous fat (Jin et al., 2010). This miRNA is also differentially expressed in murine adipocytes during differentiation (Gerin et al., 2010) and its pro-adipogenic activity is possibly through regulation of two tumour suppressor genes, SUFU and FUS-1 genes (Lee et al., 2007).

Recent studies have revealed that changes in the energy density of the diet influences gene expression in adipose tissue (Dahlman et al., 2005; Joseph et al., 2010a). However, it is not known if altering the level of dietary energy by changing lipid levels influences miRNA expression in bovine adipose tissue. We hypothesized that miRNA expression differs between adipose depots and that level of lipids in the diet alters miRNAs expression in these depots. Therefore, this research aimed to determine the miRNA expression profile from subcutaneous and visceral adipose depots of beef cattle fed diets containing high or low levels of dietary fat.

2.2 MATERIAL AND METHODS

2.2.1 Animal study and sample collections

A total of 16 Hereford x Aberdeen Angus 12 month steers were used in this experiment. They were selected based on similar body weight (456 ± 20 kg) and housed in individual pens at the Lethbridge Research Centre, Agriculture and Agri-Food Canada. Steers were fed experimental diets consisting of a total mixed ration 8:00am daily and water *ad libitum* for approximately 14 weeks. The diets fully met nutrient requirements of finishing cattle, as recommended by NRC. Diets differed in fat content, with the low fat diet estimated to have 1.95% fat, (Control group, n=8) and the high fat diet estimated to have 5.85% fat. (High fat group, n=8). Fat content of the diet was increased by including more flaxseed in the diet (Table 2.1 and Table 2.2**)**, which is an oilseed containing high amounts of alpha-linolenic acid. Experimental diets were fed until slaughter at about 15.5 months of age. Throughout the experiment several performance measures were recorded including body weight gain, feed intake, feed conversion and carcass traits including cutability, backfat thickness and adipocyte size were recorded and reported elsewhere (He et al., 2011; He et al., 2012). Subcutaneous fat (backfat) and visceral fat (perirenal fat) were collected from animal carcasses immediately after slaughter (less than 20 min), frozen in liquid nitrogen, and kept at -80°C until analyzed. The animal study was approved by the Animal Care Committee of Lethbridge Research Centre, Agriculture Agri-food Canada with ACC# 0930.

Table 2.1 Formulation of control and high fat diets

Feed Formulation	Control	High fat
Barley grain, %	85.00	75.00
Barley silage (ave 160), %	10.00	10.00
Vitamin & mineral supplement, %[1]	5.00	5.00
Flax seed, %	0.00	10.00

1 Containing the following minerals and vitamin in 1 kg: 14.67 mg copper, 58.32 mg zinc, 26.73 mg manganese, 0.66 mg iodine, 0.23 mg cobalt, 0.29 mg selenium, 4825 IU vitamin A, 478 IU vitamin D and 32 IU vitamin E

Table 2.2 Nutritional composition of control and high fat diets

Composition	Control	High fat
Dry Matter, %	73.93	74.67
Protein, %	12.71	13.81
Degr. CH2O[1]	47.60	42.00
NEm, Mcal/kg[2]	1.98	2.00
NEg, Mcal/kg[3]	1.33	1.34
Calcium, %	0.62	0.64
Fat, % (estimate)	1.95	5.85

1 Degradable carbohydrates

2 Net energy for maintenance

3 Net energy for gain

2.2.2 Measurement of adipocytes size

Fat tissues (backfat) were taken by biopsy (15 months) and were placed in warm saline and transported to the laboratory. Approximately 80 mg of tissue were cut to small pieces and fixed with 1 mL of 5% osmium tetroxide (Cartwright, 1987) for one week. After removal of the osmium tetroxide solution, fixed tissues were then placed in 8 M urea in physiologic saline to soften the tissue until most of the adipocytes could be isolated. The cells were then washed with saline and were sampled to a 24 well plate for microphotography using an inverted microscope with a digital camera. The diameter of cells was then determined by computer image analysis using of Motic Images Plus 2.0 ML software (He et al., 2010).

2.2.3 RNA extraction

Total RNA extraction was performed by homogenizing the frozen fat tissue samples with TRIZOL® (TRI reagent, Invitrogen, Carlsbad, CA, USA) following the manufacturers instructions for samples with high fat content. The concentration of total RNA was measured using the NANODROP® spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA, US) and RNA integrity was measured using the Agilent 2100 BIOANALYZER® (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). RNA with integrity number (RIN) >7.8 was used for miRNA microarray and qRT-PCR analysis.

2.2.4 Microarray analysis

miRNA profiling of adipose tissue samples from visceral and subcutaneous adipose depots from 8 cattle from each diet was performed using AGILENT 8x15K miRNA array V3 (Agilent Technologies, Santa Clara, CA, USA) customized to profile 672 bovine miRNAs based on the miRBase (Version 15). In brief, total RNA (100 ng) was firstly labeled with the AGILENT miRNA Complete Labeling and Hyb Kit (Version 2.1) by dephosphorylation with calf intestinal phosphatase, followed by denaturing and ligation with Cyanine3-pCp to the 3' end. The labeled RNA was hybridized with array slides with hybridization buffer and 10X GE blocking agent, and incubated at 55ºC for ~20 hours. Finally, the arrays were washed with GE buffers and scanned at 5 μM resolution on an Agilent G2565CA High Resolution Scanner (Agilent Technologies). Data were processed through Agilent's Feature Extraction software version 10.7.3.1 and the data was normalized to the 75th percentile using GeneSpring GX 11.5 (Agilent Technologies). Differentially expressed miRNAs were filtered by a two sample T-test with standard Bonferroni correction using the Multiple Array Viewer from Multi Experiment Viewer software (v.4.5) (Saeed et al., 2003).

2.2.5 Dataset

All the microarray data in this study are in compliance to MIAME guidelines and the data have been deposited in the publicly available NCBI's Gene Expression Omnibus Database (http://www.ncbi.nlm.nih.gov/geo/). The data are

accessible through GEO Series accession number GSE35012 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35012).

2.2.6 miRNA expression validation by qRT-PCR

Candidate miRNAs were selected based on microarray data for qRT-PCR validation. miRNA expression was carried out with TAQMAN® miRNA assays according to the manufacturer's recommendation (Applied Biosystems, Foster City, CA, USA). Briefly, cDNAs were reversely transcribed from 10 ng of total RNA using 5X specific miRNA RT primer and were amplified using a 20X TAQMAN® miRNA assay. Fluorescence signal was detected with an ABI STEPONEPLUS Real-time PCR System detector® (Applied Biosystems). A total of 28 samples from four different treatment combinations (2 diets x 2 tissue types) were used for qRT-PCR analysis, considering 7 biological replicates per group and 3 technical replicates per reaction. bta-miR-181a was selected as reference miRNA in this study due to its stable expression among all animals and treatments.

2.2.7 miRNA target prediction and functional analysis

The 12 miRNAs selected for qRT-PCR validation were further analyzed to predict which genes they may regulate. Each miRNA was individually searched in the TargetScan Release 6.0. The search was performed for mammals and customized by species (cow/*Bos taurus*). The prediction results were ranked according to context scores and site conservation (Garcia et al., 2011). The top

100 predictions for each miRNA were analyzed through IPA (Ingenuity® Systems, www.ingenuity.com). The analysis filtered genes expressed only in adipose tissue and this set of genes was submitted to a functional analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set is due to chance alone.

2.2.8 Statistical analysis

The means for performance of steers (initial and final body weight, and feed intake), fat traits (cutability, backfat thickness and adipocyte size) and average number of miRNAs per detected in each group (high fat vs. control) were compared by a two tailed two sample t-test. The miRNA expressions from qRT-PCR and its respective in microarray were tested for normality using Shapiro-Wilk test and equal variances using Levene's test, submitted to One-way ANOVA and the means were compared by Tukey test. Differences were considered statistically different at $p < 0.05$ and analyses were performed with SAS software v.9.0 (SAS Inst. Inc., Cary, NC).

2.3 RESULTS

2.3.1 Performance and fat traits of steers

Feeding steers with diets containing varying lipid content affected phenotypic traits of steers. Animals fed High fat diet had the higher body weight gain as well as the greatest feed efficiency (Table 2.3). Despite statistical

comparisons for fat traits (cutability, backfat thickness and adipocyte size) between control and high fat group there was only a trend in for steers fed the high fat diet to have lower cutability, higher backfat thickness and greater adipocyte size (Table 2.3).

Table 2.3 Performance and fat traits of animals fed control or high fat diet

	Control	**High fat**	
Performance and fat traits	Mean ± SD	Mean ± SD	p-value
Initial body weight (kg)	459.69 ± 22.31	459.06 ± 18.57	0.5445
Final body weight (kg)	604.37 ± 25.42	640.50 ± 30.65	0.0224
Weight gain (kg)	151.70 ± 20.69	181.40 ± 24.06	0.018
Feed intake – 12 wk (kg)	768.39 ± 61.48	792.15 ± 61.25	0.451
Feed Conversion Rate	5.76 ± 0.51	5.20 ± 0.30	0.019
Cutability (%)	53.25 ± 2.66	51.13 ± 1.64	0.075
Backfat thickness (mm)	17.62 ± 5.01	20.75 ± 3.54	0.171
Adipocyte size (μm)*	134.71 ± 8.95	146.50 ± 13.78	0.062

*Adipocytes used for analysis were derived from backfat tissue

2.3.2 Microarray analysis of miRNAs expression under different diets

From a total of 672 miRNA probes tested in the miRNA microarray, 244 were expressed in adipose tissue from at least one animal (Table 2.S1 - appendix). The total number of miRNAs expressed in each group of steers was 207 for control and 206 for High fat group. When the profiles of miRNA were compared,

a total of 169 miRNAs were simultaneously expressed in steers fed the high fat and control diet. A total of 75 miRNA were diet specific in subcutaneous or visceral fat, as 43 were detected only in the Control diet and 31 exclusively in the High fat diet, from subcutaneous adipose tissue, while for visceral adipose tissue, 31 miRNAs were detected only in steers fed the Control and 37 miRNAs in those fed the High fat.

The number of miRNAs detected from each steer, ranged from 115 to 162, for the Control group, and 162-163 for the High fat group. More miRNAs were detected in cattle fed the high fat diet ($p= 0.037$ in subcutaneous fat and $p= 0.002$ in visceral fat) than in those fed the control diet. Steers fed the high fat diet had a lower variability in the number of miRNA expressed than control steers (Table 2.4).

2.3.3 Microarray analysis of miRNAs expression under different fat depots

A total of 207 miRNAs were detected in both fat depots, while 37 of these were found to be tissue specific. Control steers had 15 miRNAs detected solely in subcutaneous fat and 16 solely in visceral fat. Steers fed the high fat diet had 8 miRNAs solely detected in subcutaneous fat and 27 miRNAs in visceral fat. The number of miRNAs detected among individuals in control diet was more variable than those in high fat group. There was no statistical difference in the average number of miRNAs detected in different fat depots in steers fed either diet (Table 2.4).

Table 2.4 miRNAs detected by individual and average according to diet and fat tissue

	Control diet			High fat diet	
ID	Subcutaneous fat	Visceral fat	ID	Subcutaneous fat	Visceral fat
C108	159	126	F103	163	162
C111	131	115	F109	162	163
C209	162	124	F202	162	163
C211	160	153	F204	162	162
C307	160	129	F302	163	163
C308	142	128	F303	163	162
C405	118	161	F411	162	163
C410	159	162	F412	163	163
Avg. ± SD	148.8 ± 16.6^{b}	137.2 ± 18.4^{b}		162.5 ± 0.5^{a}	162.6 ± 0.5^{a}

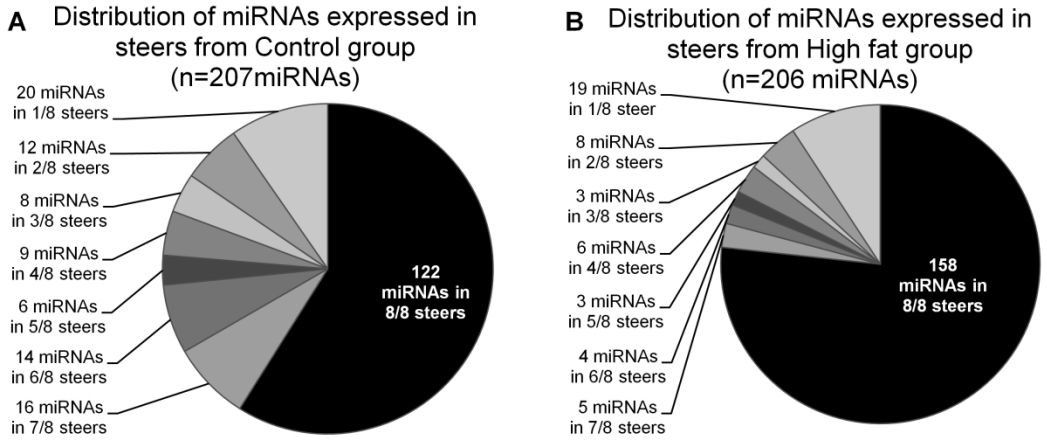
[a,b]different letters mean significant difference between diet or fat depot comparisons (subcutaneous), $p<0.05$

2.3.4 Frequency of miRNAs detected in the experimental population

The miRNAs detected varied from being present in only a single steer on each diet to being present in all eight animals sampled. For example, we detected only 118 miRNAs in subcutaneous fat tissue in a steer (ID: C405) fed the control diet while in another steer fed the same diet (ID: C209) 162 miRNAs were detected. The majority of miRNAs (58.9% in Control and 76.7% in High fat) detected were expressed in all eight animals from each treatment, while the remainder of miRNAs (41.1% in Control and 23.3% in High fat) were detected in from one up to seven animals (Figure 2.1). miRNAs detected from steers fed the

high fat diet, regardless of the depot, were more consistently detected than in steers from the Control group. A total 122 miRNAs detected in control group were conserved in all eight steers, while 158 miRNAs the from High fat group were conserved in all steers. Despite differences in miRNA profiles due to diet or tissue-specificity, a total of 83 miRNAs were simultaneously expressed in both fat depots and steers fed either the high fat or control diet.

Figure 2.1 Distribution of miRNAs detected in steers fed control diet (A) or high fat diet (B).



The distribution of miRNAs considers data from both subcutaneous and visceral fat tissue for Control (A) and High fat diet (B) groups. Each pie chart represents how the total amount of miRNAs (Control=207, High fat=206) are distributed within the animal population of each group, varying from a maximum of eight out of eight animals to a minimum of one out of eight.

2.3.5 miRNAs selected for qRT-PCR validation

Twelve miRNAs were selected for qRT-PCR validation based on miRNA microarray expression data (Table 2.S1), selecting those with differential expression ($p<0.05$) among the four experimental conditions, consisting of

combinations of two diets with two fat depots (miR-16b, -19a, -92a, -92b, -101, -103, -106, -142-5p, -196a, -296, -2368* and, -2454).

The expressions of 8 miRNAs (miR-19a, -92a, -92b, -101, -103, -106, -142-5p, and 296) were notably higher in steers fed the high fat diet than in those fed the control (Figure 2.2). Increases ranged from a low of 2.62 fold in subcutaneous adipose tissue and 8.94 fold in the visceral adipose tissue for miR-92b, to a high of 185.11 in subcutaneous adipose tissue and 968.77 fold in the visceral adipose tissue for miR-142-5p. Expression of other miRNAs was heavily influenced by fat depot. miR-2454 expression was higher in subcutaneous fat compared to visceral fat (32.20 fold in Control and 2.67 fold in high fat) whereas, conversely, miR-196a had a higher expression in visceral than subcutaneous fat (43.06 fold in Control and 17.25 fold in High fat). The miR-2368* and miR-16b also exhibited significant differences in expression, however their changes between diet types were not detected in either fat depot, with miR-16b being observed only in subcutaneous fat and miR-2368* in visceral fat (Figure 2.2). The comparison between qRT-PCR and microarray expressions showed that they were not showing the same trends for all miRNAs. Six miRNAs (miR-16b, miR-19a, miR-106, miR-142-5p, miR-196a and miR-2454) had the expression in agreement between qRT-PCR and microarray, however the other six (miR92a, miR-92b, miR-101, miR-103, miR-296 and miR-2368*) had divergent results.

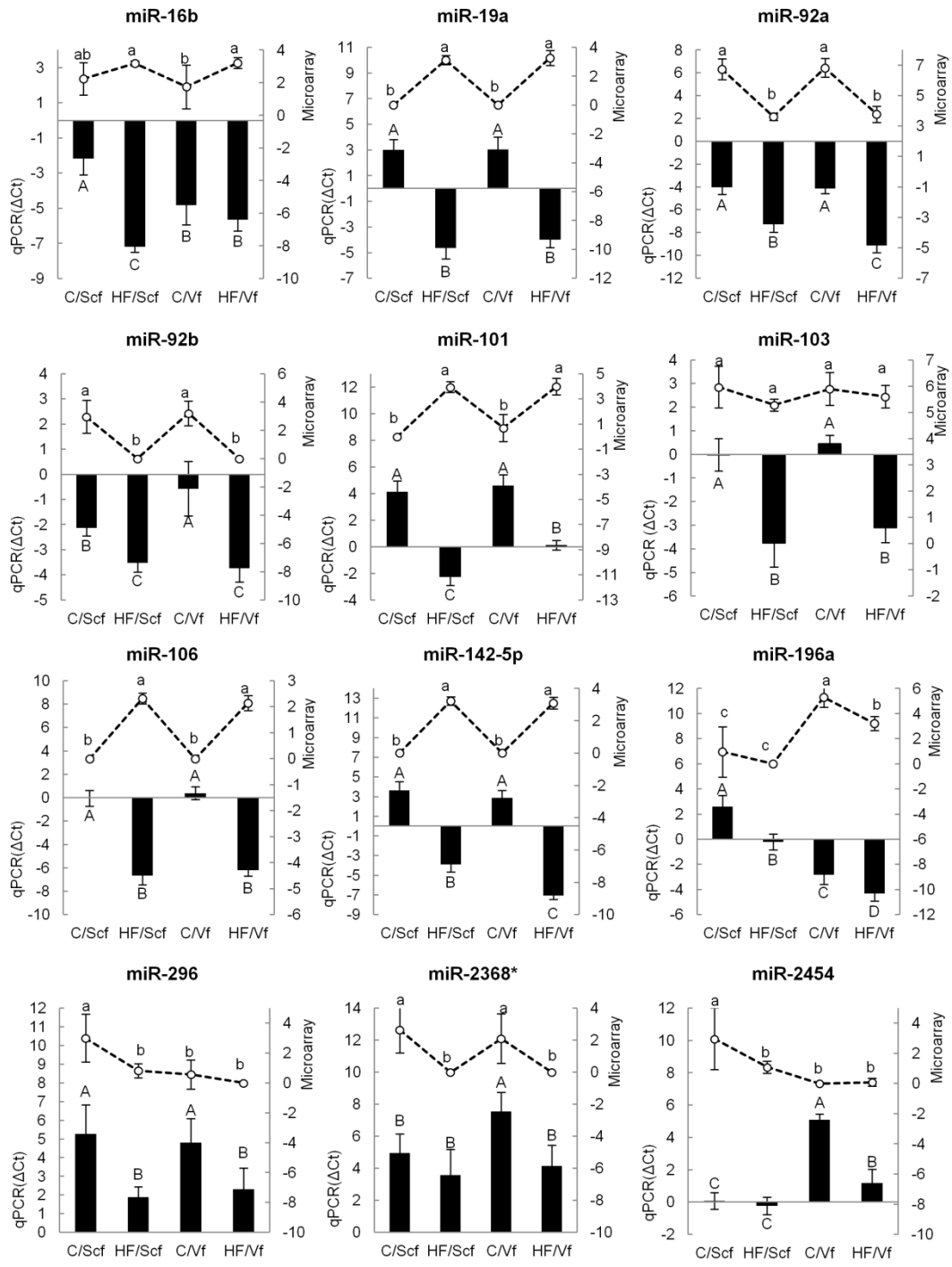


Figure 2.2 Expression of miRNAs by qRT-PCR and microarray.

MicroRNA expression is presented for the following treatments on the horizontal axis: Control

diet/Subcutaneous fat (C/Scf), High fat diet/Subcutaneous fat (HF/Scf), Control diet/Visceral fat (C/Vf) and High fat diet/Visceral fat (HF/Vf). The graphs show the miRNA expression from miRNA microarray represented by lines (--○--) on the top and values are shown on the left vertical axis as normalized intensity values. Quantitative PCR expression is represented using columns (▬) on the bottom and values are shown on the right vertical axis as delta cycle threshold (ΔCt). A, B, C, D Columns (qPCR) with different letters differ significantly ($p<0.05$). a, b, c Markers in lines (microarray) with different letters differ significantly ($p<0.05$). Data are presented as Mean ± Standard deviation.

2.3.6 miRNA predicted targets and functional analysis

A total of 34 unique genes expressed in adipose tissue having functions related to lipid metabolism or adipogenesis were predicted to be targets of the 12 miRNAs quantified by qRT-PCR (Table 2.5). The number of potential gene targets varied among miRNAs with a high of seven predicted genes for miR-101 and miR-2368* and a low of one predicted gene for miRNA-196a. The majority of the predicted genes (28 out of 34) were predicted targets of only one of the 12 miRNAs analyzed, while six of them (ABHD5, ADRB1, CLOCK, PPARGC1B, REST and SGK1) were predicted targets for two miRNAs. The functional analysis identified the biological functions relevant to the 34 predicted target genes (Table 2.6). A total of 25 biological functions were relevant to adipose tissue physiology, with 24 classified in the Lipid Metabolism category and 1 in the Connective Tissue Development and Function category. Some biological functions were more prevalent than other, such as synthesis of lipids which accounted for 19 of the 34 predicted genes.

Table 2.5 Predicted miRNA gene targets with functions related to lipid metabolism and/or adipogenesis

microRNA	Symbol	Entrez Gene Name
bta-miR-16b	FGF2	fibroblast growth factor 2 (basic)
	GNAI3	guanine nucleotide binding protein (G protein),alpha inhibiting activity polypeptide 3
	LRP6	low density lipoprotein receptor-related protein 6
		platelet-activating factor acetylhydrolase 1b,
	PAFAH1B2	catalytic subunit 2 (30kDa)
	SMAD7	SMAD family member 7
	WNT3A	wingless-type MMTV integration site family, member 3A
bta-miR-19a	SOCS3	suppressor of cytokine signaling 3
	SGK1	serum/glucocorticoid regulated kinase 1
	ADRB1	adrenergic, beta-1-, receptor
	ABHD5	abhydrolase domain containing 5
bta-miR-92a,b	ADRB1	adrenergic, beta-1-, receptor
	TEF	thyrotrophic embryonic factor
bta-miR-101	SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporters), member 2
	SGK1	serum/glucocorticoid regulated kinase 1
	PRKCE	protein kinase C, epsilon
	PPARGC1B	peroxisome proliferator-activated receptor gamma, coactivator 1 beta
	KITLG	KIT ligand
	GSK3B	glycogen synthase kinase 3 beta
	APP	amyloid beta (A4) precursor protein
bta-miR-103	BDNF	brain-derived neurotrophic factor
	CLOCK	clock homolog (mouse)
		prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and
	PTGS2	cyclooxygenase)
bta-miR-106	ABHD5	abhydrolase domain containing 5
	REST	RE1-silencing transcription factor
bta-miR-142-5p	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1
	ACSL6	acyl-CoA synthetase long-chain family member 6
	CAV2	caveolin 2
	REST	RE1-silencing transcription factor
bta-miR-196a	GLTP	glycolipid transfer protein
bta-miR-296	ABHD4	abhydrolase domain containing 4
	PPARGC1B	peroxisome proliferator-activated receptor gamma, coactivator 1 beta
bta-miR-2368*	ACSL3	acyl-CoA synthetase long-chain family member 3
	CARM1	coactivator-associated arginine methyltransferase 1
	CLOCK	clock homolog (mouse)
	FOXO1	forkhead box O1
	LIF	leukemia inhibitory factor (cholinergic differentiation factor)
	PPARA	peroxisome proliferator-activated receptor alpha
	SNCA	synuclein, alpha (non A4 component of amyloid precursor)
bta-miR-2454	B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1
	PDGFB	platelet-derived growth factor beta polypeptide

Table 2.6 Functional analysis of gene targets involved in lipid metabolism and adipogenesis

Category	Function	Pathways	p-value	Predicted gene targets involved
Lipid Metabolism	synthesis	synthesis of lipid	2.15E-15	ABCA1, ABHD5, ACSL3, ACSL6, APP, B4GALT1, BDNF, CAV2, FGF2, FOXO1, GNAI3, KITLG, LIF, PDGFB, PPARA, PTGS2, REST, SNCA, SOCS3
		synthesis of phospholipid	1.68E-06	ABHD5, ACSL6, FGF2, PDGFB, PTGS2, SOCS3
		synthesis of fatty acid	2.07E-06	ABCA1, ACSL3, APP, CAV2, KITLG, LIF, PTGS2, SNCA
		synthesis of steroid	3.27E-06	APP, BDNF, FGF2, KITLG, LIF, PPARA, REST
	metabolism	metabolism of membrane lipid derivative	7.67E-13	ABCA1, ABHD5, ACSL6, APP, B4GALT1, BDNF, FGF2, GNAI3, KITLG, PDGFB, PTGS2, SNCA, SOCS3
		fatty acid metabolism	3.81E-12	ABCA1, ACSL3, ACSL6, APP, B4GALT1, CAV2, FGF2, GLTP, GNAI3, KITLG, LIF, PPARA, PTGS2, SNCA, TEF
		metabolism of phospholipid	3.44E-07	ABHD5, ACSL6, FGF2, PDGFB, PTGS2, SNCA, SOCS3
		metabolism of acylglycerol	5.68E-06	ABHD5, ACSL6, FOXO1, GNAI3, KITLG
	quantity	quantity of lipid	3.73E-11	ABCA1, ADRB1, APP, B4GALT1, BDNF, FOXO1, KITLG, LIF, PPARA, PPARGC1B, PRKCE, PTGS2, SGK1, SLC12A2, SNCA
		quantity of steroid	5.85E-10	ABCA1, ADRB1, APP, BDNF, LIF, PPARA, PPARGC1B, PRKCE, PTGS2, SGK1, SLC12A2
		quantity of triacylglycerol	5.39E-06	BDNF, FOXO1 (includes EG:2308), PPARA, PPARGC1B, PRKCE, PTGS2
		quantity of sterol	7.10E-06	ABCA1, APP, BDNF, PPARA, PPARGC1B, PTGS2
	oxidation	oxidation of lipid	2.84E-08	ABHD5, ACSL3, ACSL6, APP, PPARA, PPARGC1B, PTGS2, SNCA
		oxidation of fatty acid	1.84E-06	ABHD5, ACSL3, ACSL6, PPARA, PPARGC1B, PTGS2
		oxidation of oleic acid	3.36E-06	ACSL3, ACSL6, PPARA
	accumulation	accumulation of lipid	2.47E-07	ABCA1, ABHD5, ACSL6, APP, FOXO1, GSK3B, PAFAH1B2, PPARA
	release	release of lipid	2.97E-07	ABCA1, APP, CAV2, GNAI3, KITLG, PDGFB, PRKCE, PTGS2
		release of fatty acid	1.32E-05	CAV2, GNAI3, KITLG, PDGFB, PRKCE, PTGS2
	cleavage	cleavage of lipid	3.36E-07	ABHD4, ABHD5, GNAI3, PAFAH1B2, PPARA, PTGS2, SNCA
	biosynthesis	biosynthesis of glycosphingolipid	2.25E-06	APP, B4GALT1, FGF2, GNAI3, KITLG
	concentration	concentration of lipid	3.89E-06	ABCA1, APP, CLOCK, PPARA, PTGS2
	hydrolysis	hydrolysis of lipid	4.63E-06	ABHD4, ABHD5, GNAI3, PAFAH1B2, PPARA, SNCA
	esterification	esterification of lipid	5.68E-06	ABCA1, APP, LIF, PPARA
	steroidogenesis	steroidogenesis	1.39E-05	APP, BDNF, FGF2, KITLG, LIF, PPARA
Connective Tissue Development and Function	differentiation	differentiation of adipocytes	1.28E-10	ADRB1, CARM1, FOXO1 (includes EG:2308), GSK3B, LIF, LRP6, PPARA, SMAD7, WNT3A

2.4 DISCUSSION

Diet is known to impact many phenotypic traits in ruminants such as growth rate, meat and the fat content and fatty composition of milk and meat. Steers fed the high fat diet (5.85%) in the present study had higher weight gain and improved feed efficiency compared to those fed the control diet, with some of these differences likely being attributable to differences in adipose metabolism as suggested by changes in carcass cutability, backfat thickness and adipocyte size. Recent advanced functional genomics approaches have allowed the identification of the mechanisms of adipogenesis at gene expression level (Jin et al., 2012) and their alteration in response to different diets (Joseph et al., 2010a; Joseph et al., 2010b). A recent study showed that beef steers supplemented with a high fat supplement (0.31Kg/day of corn oil) presented a higher lipogenic activity in fat tissue, with higher expression of fatty acid synthase and stearoyl-CoA desaturase (Joseph et al., 2010a). The increase of adipose mass by high fat diets can also enhance the production of adipokines, influencing the regulation not only of fat tissue, but also promoting systemic effects on metabolism (Rosen and Spiegelman, 2006; Galic et al., 2010). In this study, we further investigated the molecular regulation of beef cattle adipose tissue by characterizing the expression of miRNAs in adipose tissue.

miRNAs are known to regulate gene expression by binding to complementary sequences of their target messenger RNAs which leads to translational repression and affects several physiological processes, including adipogenesis (Kim et al., 2009; Romao et al., 2011). miRNAs from adipose tissue

exhibited differential expression levels between steers of distinct breed as well as with different levels of backfat thickness (Jin et al., 2010). A recent study further showed variation of miRNA profiles among different locations of subcutaneous backfat (Jin et al., 2009). Our current study is the first to report that the miRNA expression profiles in steers differed significantly in two fat depots in response to varying dietary fat content. Our results are in agreement with another study that showed the manipulation of dietary fat (varying levels of conjugated linoleic acid in the diet) impacted the expression of miRNAs (miR-143, -107, -221 and -222) from retroperitoneal adipose tissue of mice (Parra et al., 2010). Although the miRNA expression was altered due to dietary manipulation in both species; mice and bovine have different molecular mechanisms of adipogenesis, (e.g. different patterns of C/EBPα expression in adipocytes differentiated *in vitro*) (Taniguchi et al., 2008; Dodson et al., 2010), which might also happen in live animals and in part, differences in adipogenesis might be due to variation in the fatty acid profile presented to adipocytes as a result of differences in microbial biohydrogenation in the intestinal tract.

Microarray analysis revealed that most miRNAs were simultaneously expressed in steers fed both diets, but a parcel of miRNAs was specific to each diet which might tailor the regulation of adipose tissue to the specific metabolic requirements imposed by each diet. Dietary fat changes alter gene expression (mRNA level) in bovine adipose tissue (Joseph et al., 2010b) and considering that miRNA is a major regulator on gene expression, it is perhaps not unexpected that distinct miRNA profiles in adipose tissue are associated with changes in the fat

content of the diet. Steers fed the high fat diet had a higher average number of miRNA expressed compared to control steers (Table 2.4), suggesting that a high fat diet increased the complexity of the role of miRNAs in regulating gene expression. We speculate that it is likely that more pathways associated to fat metabolism and development are activated in the adipose tissue of high fat diet animals, therefore more miRNAs are needed to regulate fat development in animals fed high fat diet. However, the differences in miRNA number between steers from control and high fat groups may also suggest that animals in either group have different response to the environment effect or interactions between genetic regulation and environment influence. Future studies to investigate the diet effects on the same animals are necessary to clarify if the expression of microRNAs is directly regulated by the diet.

Two parameters can differentiate the miRNA profile between individuals. One is the presence or absence of distinct miRNAs and the other is the level of expression of each miRNA. However it is expected that individuals should express a core number of miRNAs fundamental for the regulation of genes involved in adipose metabolism. Steers fed the high fat diet presented a larger core (76.7%) of miRNAs (expressed in all eight animals) compared to control steers (58.9%), suggesting that a high fat diet demands an increased regulational miRNA core to regulate adipose tissue metabolism. Among those, we detected well studied miRNAs including miR-103 which has a pro-adipogenic role (Xie et al., 2009a), miRNA let-7, reported to have an anti-adipogenic role in 3T3-L1 adipocyte cell culture regulating HMGA2 (Sun et al., 2009) and miR-27b which

regulates the expression of PPARγ, considered the master regulator of adipogenesis (Karbiener et al., 2009). These findings suggest that miRNA based regulation in adipogenesis is common in different mammalian species. It is noticeable that the first bovine specific miRNAs identified may be associated with bovine adipogenesis. For example, miR-2368* and miR-2454 are two bovine specific miRNAs (Glazov et al., 2009). Although their functions are unknown, based on bioinformatics prediction tools they may regulate mRNAs belonging to peroxisome proliferator-activated receptor alpha (PPARA) and platelet-derived growth factor beta polypeptide (PDGFB) genes. Future studies are necessary to understand their roles in visceral fat in response to different diets.

The qRT-PCR analysis further verified the diet effect on expression of twelve miRNAs, suggesting that that these miRNAs may be involved in regulation of specific functions or pathways in bovine adipose tissue. It is known that various miRNAs regulate adipogenesis (O'Rourke et al., 2006; Song and Tuan, 2006; Williams, 2008; Alexander et al., 2011; Romao et al., 2011). Up to date, the miRNA expression has been widely compared among different species and tissue types. A study on humans showed that miR-103 was the most stable miRNA under different variable conditions, including fat tissue location, body mass index, physiological vs. pathological states, and gender (Neville et al., 2011). However, this study did not examine the effect of diet on miRNA expression. In the present study, miR-103 was upregulated in steers fed high fat diet suggesting that a change in dietary fat content may alter the miR-103 expression and play a regulatory role in bovine adipose tissue. This miRNA was

found to bind mRNA of caveolin-1, a factor that regulates insulin sensitivity, with the upregulation of miR-103 in liver or fat tissue being associated with impaired glucose homeostasis in mice (Trajkovski et al., 2011).

In addition, an upregulation of members of the cluster miR-17-92 (miR-19a and -92a) in adipose depots of steers fed high fat diet compared to control was also observed, suggesting an increased adipogenesis in these animals. The miRNA cluster miR-17-92 was shown to be pro-adipogenic as overexpression of miR-17-92 accelerated adipocyte differentiation and increased triglyceride accumulation in 3T3-L1 cells through inhibition of Rb2/p130, a key cell cycle regulator and tumor suppressor (Wang et al., 2008). Similarly, expression of other miRNAs (miR-92b, -101, -106, -142-5p, -296) was higher in both fat depots in steers fed high fat diet as compared to control steers, but the regulatory role of these miRNAs remains to be defined in adipose tissue.

To date, the molecular mechanisms of bovine adipogenesis in different adipose depots have not been well defined. Serial analysis of gene expression found a total of 82 genes up or downregulated (>2 fold change) depending on the fat depot (subcutaneous vs. intramuscular fat) in Korean cattle (Bong et al., 2010). Differences in gene expression among different fat depots have also been observed in pig and mouse (Hishikawa et al., 2005). In this study, we found miRNAs influenced mainly by adipose depot location, such as miR-196a, which was highly expressed in visceral fat while miR-2454 was highly expressed in subcutaneous fat. The adipose depot specificity of these miRNAs is likely related to the regulatory physiological peculiarities of each fat depot. miRNAs tissue

specificity has already been reported in other species including mice (Lagos-Quintana et al., 2002b; Gao et al., 2011) and human (Liang et al., 2007) indicating the need of different regulatory mechanisms to address the unique physiology of each tissue type.

Furthermore, it is notable that both microarray and qRT-PCR revealed a significant difference of expression of miR-16 in steers fed the two diets. This miRNA has been widely used as an endogenous control miRNA for qRT-PCR analysis (Davoren et al., 2008; Jin et al., 2010; Mortarino et al., 2010; Zhao et al., 2010). Its expression was not consistent in subcutaneous fat across the two diets. Indeed, miR-16 is reported to regulate a gene involved in apoptosis, B cell lymphoma 2 (BCL2, anti-apoptotic gene) (Cimmino et al., 2005), suggesting miR-16 has pro-apoptotic function. Considering that apoptosis is reported to be diet influenced in adipose tissue and associated with obesity (Alkhouri et al., 2010), we can assume that higher miR-16 levels in steers fed high fat was also diet mediated. This indicates that miR-16 is not a stable endogenous control for adipose tissue across diets that differ in fat level. Therefore, future studies also considering the impact of dietary lipid levels on miRNAs will be needed to identify an alternative endogenous control.

The twelve miRNAs analyzed by qRT-PCR had at least one predicted gene target with functions related to lipid metabolism and/or adipogenesis. These findings suggest that these differentially expressed miRNAs may play a role in the regulation and development of bovine fat tissue. Some of these miRNAs may even play a broader role such as miR-101 and miR-2368* which are predicted to

regulate 7 genes related to lipid metabolism and/or adipogenesis (Table 2.5), including peroxisome proliferator activated receptor alpha (PPARα), an important regulator of energy homeostasis in white adipose tissue (Goto et al., 2011). The functional analysis indicates that the predicted genes filtered according to their presence in adipose tissue have a broad role in lipid metabolism and adipogenesis. The processes they are involved range from synthesis to release of lipids, indicating the important role miRNAs may have on adipose tissue fat metabolism. A previous study using digital gene expression tag profiling showed that genes involved in adipogenesis such as FGF2, GNAI3, LRP6, PAFAH1B2, SMAD7, WNTA3, SOCS3, SGK1, ADRB1, ABHD5, SLC12A2, PRKCE, KITLG, APP, PTGS2, ABCA1, ACSL6, CAV2, ABHD4, LIF, PPARA, SNCA, B4GALT1 and PDGFB were expressed in subcutaneous adipose tissue of beef cattle (Jin et al., 2012), which belong to the predicted miRNA targets in this study.

It is a complex task to determine the exact role of miRNAs in the gene regulation of adipose as they do not require a perfect complementarity to regulate target messenger RNA (Wahid et al., 2010). Complementarity is the main feature that miRNA target prediction tools use to find miRNA targets, as a result they may find hundreds of possible targets for one miRNA (Saito and Sætrom, 2010). Additional studies to experimentally identify the targets of miRNAs differentially expressed in fat tissue will be fundamental to improve our understanding of the roles of miRNAs in gene regulation in adipose tissue under a range of dietary conditions. In addition, the fat tissues collected in this study consist of a mixture of different cell types. Therefore future studies using bovine adipocyte cell lines

may further verify the essential regulatory functions of miRNA in bovine adipogenesis.

It is noticeable that miRNAs have also been reported to be part of the regulatory mechanisms of epigenetics. Several DNA methyltransferase genes (DNMTs), which encode proteins responsible for establishment and maintenance of the methylation of the fifth carbon of cytosine residues in DNA, are reported to be targets of miRNAs (Denis et al., 2011). Moreover, studies show that DNA methylation and histone modifications not only regulate the expression of protein coding genes, but also of miRNAs (Sato et al., 2011). Therefore it is evident the miRNA impact on epigenetic regulatory mechanisms and vice versa. Their interplay modulates gene expression at transcriptional and post-transcriptional levels with implications to the regulation of global gene expression and potentially to adipogenesis. Besides, recently the miR-483-3p was reported to be epigenetically regulated by nutrition during early life in mice and humans with impacts on the regulation of adipose tissue in adult life (Ferland-McCollough et al., 2012).

In conclusion, the results obtained from this study revealed that the expression of miRNAs differed between subcutaneous and visceral fat depots, suggesting that the molecular mechanism of adipogenesis is site dependent in beef cattle. Our study further identified significant changes in the types and level of expression of miRNAs in steers fed diets differing in fat content. These findings suggest that miRNAs serve as regulators of adipogenesis in response to different dietary conditions. High fat diets have been considered as one of the main factors

causing obesity in humans. The identification of the factors that alter miRNAs expression could expand current understanding of the environment and genetic factors that influence gene expression during adipogenesis. Significant individual variation of miRNA expression within the same diet group has been observed. Future studies to link the genotypes to variation of miRNA and their gene targets expressions are essential to elucidate the roles of miRNAs in fat adipogenesis.

2.5 APPENDIX

Table 2.S1 Expression of miRNAs detected by miRNA microarray

http://www.plosone.org/article/fetchSingleRepresentation.action?uri=info:doi/10.1371/journal.pone.0040605.s001

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3 Elucidation of molecular mechanisms of physiological variations between subcutaneous and visceral fat depots under different nutritional regimes [6]

3.1 INTRODUCTION

In the past decade, adipose tissue has received increasing attention since fat not only aids in the regulation of energy balance, but also plays an important role in endocrine function (Galic et al., 2010; Poulos et al., 2010). Adipose tissue dysfunction has also become an important health concern and obesity is now considered as an epidemic condition (Catenacci et al., 2009) with more than 1.5 billion people worldwide being overweight or obese (Nguyen and Lau, 2012). One of the concerns in the development of obesity is the increase of the consumption of high fat foods; therefore efforts have been made to increase the leanness of beef (Wang and Beydoun, 2009; McNeill et al., 2012). Meat is an important protein source and the world meat consumption is projected to increase by 3.2kg/per person per year until 2021 (OECD-FAO, 2012). Since fat is an important component in animal productivity and meat quality (Hausman et al., 2009; Dodson et al., 2010), it is necessary to improve our understanding on regulation of adipogenesis in beef in order to provide meat with lipid profiles that are desirable for human consumption.

[6] A version of this chapter has been published. Romao, J.M.; Jin, W.; He, M.; McAllister, T.; Guan, L.L. 2013. Elucidation of molecular mechanisms of physiological variations between subcutaneous and visceral fat depots under different nutritional regimes. PLoS ONE 8(12): e83211. doi:10.1371/journal.pone.0083211.

Adipogenesis is an essential biological process in mammals, which involves the development of mature adipocytes from preadipocytes (Large et al., 2004). This process modulates the adiposity of individuals and can be influenced by various factors such as diet, fat depot, age and genetics (Kirkland et al., 2002; Hosooka et al., 2008; Jin et al., 2012). Adipogenesis is regulated by transcription factors such as peroxisome proliferator-activated receptor gamma (PPARγ), members of CCAAT/enhancer binding proteins, kruppel-like factors, and sterol regulatory element-binding proteins (SREBP), which control the expression of adipogenic genes that participate in the differentiation of adipocytes (Lefterova and Lazar, 2009; White and Stephens, 2010).

Up to date, our understanding on molecular regulation of adipogenesis is based mainly on information obtained from gene expression studies at the mRNA level (Basu et al., 2012). However, transcript levels do not necessarily correlate well with protein expression (Gry et al., 2009; Ghazalpour et al., 2011; Schwanhausser et al., 2011) and therefore phenotype.

Recently, it has become evident that not only transcription factors but also microRNA (miRNAs) (Xie et al., 2009b; Romao et al., 2011; Romao et al., 2012) and epigenetic mechanisms are implicated in the regulation of adipose tissue metabolism (Ferland-McCollough et al., 2012). Post-transcriptional regulatory mechanisms such as RNA interference as carried out by miRNAs or small RNAs may repress the translation of mRNAs into proteins (Ebert and Sharp, 2012). As the correlation between mRNA levels and protein expression is moderate (Gry et

al., 2009; Ghazalpour et al., 2011; Schwanhausser et al., 2011), characterization of translated proteins may be a better predictor of phenotype.

To date, few studies have attempted to study the regulation of bovine adipogenesis at the protein level. Using 2D electrophoresis and MS analysis, 13 differentially expressed proteins were identified between steers with high or low subcutaneous fat thickness (Zhao et al., 2010b). Differentially expressed proteins were also identified between preadipocytes and mature adipocytes, and also among adipocytes from different fat depots (omental, subcutaneous and intramuscular) (Rajesh et al., 2010). However, the influence of diet and fat depots on the proteome of bovine adipose tissue *in vivo* has not been investigated. Therefore, the aim of our study was to characterize the proteomic profile of subcutaneous and visceral adipose tissues of beef steers fed a high or a low fat level diet and to identify the potential regulatory molecular mechanisms of protein expression.

3.2 MATERIAL AND METHODS

3.2.1 Animal study and sample collection

A total of 16 Hereford x Aberdeen Angus steers (12 months old) were used in this experiment. Steers were selected based on similar body weight (456 ± 20 kg) and housed in individual pens at the Lethbridge Research Centre. They received feed and water *ad libitum.* The steers were fed experimental diets for approximately 14 weeks. The control diet contained 2.7% fat (Control group, n=8) and the high fat diet contained 7.1% fat (High fat group, n=8). Fat content

was increased by including 10% flaxseed in the diet as described in Table S3.4. Throughout the experiment several performance measures were recorded including body weight gain, feed intake, feed conversion ratio and carcass traits including cutability, backfat thickness and adipocyte size. Growth performance results are reported elsewhere by He et al. (He et al., 2012a; He et al., 2012b). Adipose tissue samples were collected from animal carcasses immediately after slaughter (less than 20 min), frozen in liquid nitrogen, and kept at -80°C until analyzed. Subcutaneous fat was collected from the backfat depot, close to the region of the last thoracic vertebrae and visceral fat was collected around the kidneys. The study was approved by the Animal Care Committee of Lethbridge Research Centre, Agriculture Agri-food Canada with ACC# 0930.

3.2.2 Measurement of adipocytes size

Subcutaneous adipose tissue was collected by biopsy (15 months), with a portion of the sample placed in warm saline solution and transported to the laboratory and processed immediately after sampling. Tissues were cut into small pieces of approximately 80 mg and fixed with 1 mL of 5% osmium tetroxide (Cartwright, 1987). After removal from the osmium tetroxide solution, fixed tissues were placed in 8 M urea in physiologic saline (NaCl 0.9%) to soften the tissue in order to isolate adipocytes. The cells were then washed with saline and transferred to a 24 well plate for microphotography using an inverted microscope (Olympus CKX41, Olympus, Japan) with a digital camera (Moticam 2300, Motic China Group Co., Ltd., China). The diameter of cells was determined by computer

image analysis using the software Motic Images Plus 2.0 ML as described by He et al (He et al., 2010).

3.2.3 Protein extraction and total protein quantification

Tissue samples stored at -80°C were ground using liquid nitrogen. Protein extraction was performed for each sample by homogenizing 100 mg of ground adipose tissue with 1mL of 2-D protein extraction buffer-V with diluent II (Urea (< 8 M), Thiourea (< 5 M), and CHAPS (< 10%) (GE Healthcare, Uppsala, Sweden) added with DTT (40mM), using a Precellys®24 tissue homogenizer (Bertin Technologies, Saint Quentin, France). The homogenate was centrifuged at 17,000 x g for 30 min at 4°C and the supernatant was transferred to new tubes avoiding the lipid layer formed. Total protein quantification was performed after extraction using RC DC (reducing agent compatible and detergent compatible) Protein Assay based on Lowry method (Bio-Rad, Hercules, CA, USA) according to manufacturer instructions in order to ensure adequate protein quantity for downstream applications.

3.2.4 Label free LC-MS/MS quantification

Thirty µg of total protein from each sample was subjected to Label-free quantification LC-MS/MS (Old et al., 2005) at the Mass Spectrometry (MS) and Proteomics Resource of the W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University. Details on LC-MS/MS analysis are described in Protocol S1. Briefly, the proteins were firstly precipitated using

methanol/chloroform and dissolved in 8M urea/0.4M ammonium bicarbonate (pH=8.0) and DTT (45mM), incubated at 37°C for 20 min and cooled to room temperature. Following incubation with 100mM iodoacetamide (IAN) for 20 min. The protein samples were digested with 2µg of Lys C incubated for 5 h, followed by 2µg of trypsin and incubated overnight at 37°C. LC-MS/MS was performed on a LTQ Orbitrap XL (Thermo Scientific, Waltham, MA, USA) equipped with a Waters nanoAcquity UPLC system and used a Waters Symmetry® C18 180µm x 20mm trap column and a 1.7 µm, 75 µm x 250 mm nanoAcquity™ UPLC™ column (35ºC) for peptide separation. A total of 0.2ug of sample per run was used and samples were randomized with 2 blanks after each run. Each sample was run in duplicate. LTQ Orbitrap XL acquired MS using 1 microscan, and a maximum inject time of 900ms followed by three data dependant MS/MS acquisitions in the ion trap (Bordner et al., 2011) with a total cycle time for both MS and MS/MS acquisition of 2.4 sec. Data analysis was done using Progenesis LC-MS software (Nonlinear Dynamics Ltd., New Castle, U.K) (www.nonlinear.com). First, the acquired spectra were imported to the software. One sample run was selected as a reference while the others were automatically aligned to that run to minimize retention time variability between runs. All runs were selected for detection with an automatic detection limit. A normalization factor was then calculated for each run to account for differences in sample loads among injections. The MSMS were exported for Mascot database searching and results imported into the Progenesis LCMS software, where search hits were assigned to corresponding features.

3.2.5 Database search and protein identification

The data were processed with Progenesis LCMS which provided the .mgf files that were searched using Mascot search algorithm version 2.2.0. (Matrix Science Inc., London, U.K.) (Hirosawa et al., 1993) to identify proteins. The data was searched using the Uniprot database (http://www.uniprot.org), bovine taxonomy. The following search parameters were used: type of search (MS/MS Ion Search), enzyme (trypsin), variable modifications (carbamidomethyl (Cys), oxidation (Met)), mass values (monoisotopic), protein mass (unrestricted), peptide mass tolerance (± 25 ppm), fragment mass tolerance (± 0.6 Da), charge (+7), maximum missed cleavages (3), decoy (yes), and instrument type (ESI-TRAP).

3.2.6 RNA extraction

Total RNA was extracted from frozen (-80°C) ground adipose tissue. Homogenization of the frozen fat tissue samples was performed using a Precellys®24 tissue homogenizer with TRIZOL® (TRI reagent, Invitrogen, Carlsbad, CA, USA) following the manufactures instructions for samples with high fat content. The concentration of total RNA was measured using the NANODROP® spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA, US) and RNA integrity was measured using the Agilent 2100 BIOANALYZER® (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). RNA with integrity number (RIN) > 7.8 was used for qRT-PCR analysis.

3.2.7 mRNA expression validation by qRT-PCR

Candidate mRNAs were selected based on label free protein quantification data for qRT-PCR validation. First strand was obtained from total RNA for each sample using random primers and reverse transcription reagents (Invitrogen, Carlsbad, CA, USA) according to manufacturer's guidelines. Each PCR reaction (20µL) consisted of 2ng of template cDNA, 2× SYBR Green I Master Mix buffer (10 µL, Applied Biosystems, Foster City, CA), and 300 n*M* forward and reverse primers.

Fluorescence signal was detected with an ABI STEPONEPLUS Real-time PCR System detector® (Applied Biosystems) using the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. A total of 28 samples, 7 samples from each fat depot – diet combination were used for qRT-PCR analysis, with a total of 3 technical replicates per reaction. Beta-actin was used as reference gene in this study due to its stable expression among all animals and treatments. Primers were manufactured by Invitrogen and sequences are shown in table S3.5. Gene expression was analyzed by relative quantification (delta delta Ct method).

3.2.8 Bioinformatics analysis

Functional analysis for all proteins and DE proteins was performed using Ingenuity Pathway Analysis Package (IPA). Molecules from the dataset were mapped to the Ingenuity Knowledge Base (http://www.ingenuity.com) and associated with biological functions through a functional analysis using human

and mice as background. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set was relevant. A p-value < 0.05 indicated that the function was significant to the dataset. The Downstream Effects Analysis (IPA®) was based on proteins differentially expressed between diets x depot combinations and determined if a biological function increased or decreased based on which proteins were involved along with their expression values. Z-scores >2 or <-2 indicated that the activity of a relevant function was significantly increased or decreased. Upstream regulator analysis aimed at predicting which upstream regulators (e.g. transcription factors) that control the expression of a set of genes. This analysis was based on genes coding DE proteins between fat depots and transcription factors predicted with $p<0.05$ were relevant to the dataset. Activation status of TFs (increased or decreased expression) was based on the expression of the proteins involved and significance was represented as Z-score. Significance statements for data analyzed through IPA (Ingenuity® Systems, www.ingenuity.com) in functional analysis (p-value), downstream effect analysis (z-score), and upstream regulator analysis (p-value and Z-score) were calculated based on IPA algorithms.

3.2.9 Statistical analysis

The data on protein expression was tested for normality using Shapiro-Wilk test and equal variances using Levene's test. Then, effects of fat depot, diet and their interaction (fat depot x diet) on protein expression were measured

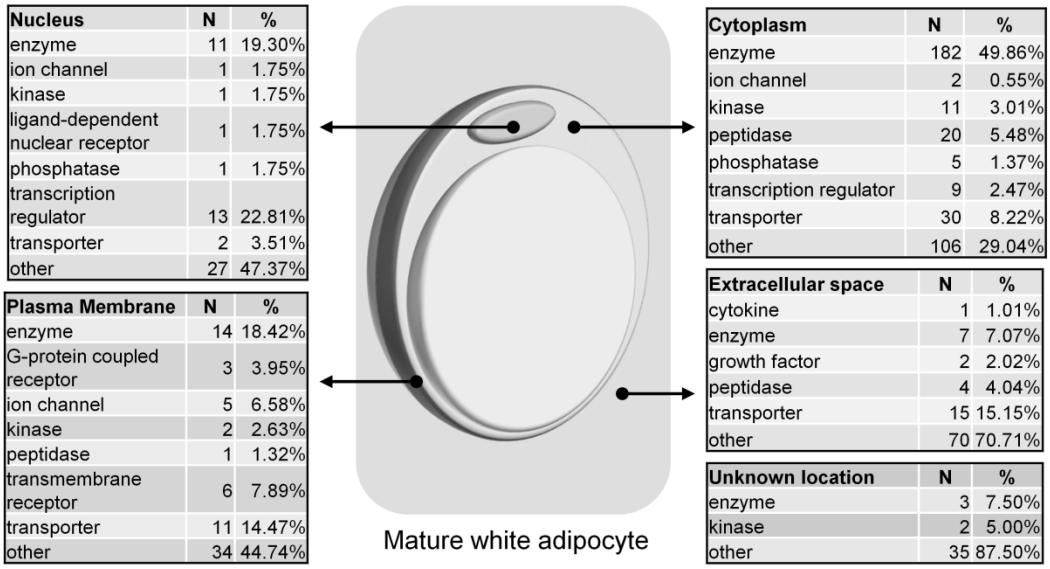
through two-way ANOVA with significance level at $p < 0.01$. Proteins differentially expressed between treatments were selected based on least square means from two-way ANOVA ($p<0.05$) and a fold change > + 1.5 or < -1.5. Correlations between individual measured fat traits (subcutaneous fat thickness and adipocyte size) and individual protein expression were calculated using Pearson correlation coefficient (R) with significance defined at $p < 0.05$. The same was applied to correlations between mRNA and protein expression. Statistical analysis was performed with SAS software v.9.0 (SAS Inst. Inc., Cary, NC).

3.3 RESULTS

3.3.1 Location and types of proteins expressed in bovine adipose tissue

A total of 682 proteins were identified and quantified with at least one unique peptide in all experimental groups: Control (2.7% fat) diet/Subcutaneous adipose tissue (C.SAT), Control diet/Visceral adipose tissue (C.VAT), High fat (7.1% fat) diet/Subcutaneous adipose tissue (HF.SAT) and High fat diet/Visceral adipose tissue (HF.VAT). Among these proteins, 637 were classified into a main protein category based on their function and into a cellular location including the nucleus (n=57), cytoplasm (n=365), plasmatic membrane (n=76), extracellular space (n=99) or unknown location (n=40) according to the Ingenuity Knowledge Base using Ingenuity Pathway Analysis Software Package (IPA®) (Figure 3.1). The function and/or location of the remaining proteins in our study could not be

annotated, and therefore only these 637 proteins were subjected downstream analysis.



Nucleus	N	%
enzyme	11	19.30%
ion channel	1	1.75%
kinase	1	1.75%
ligand-dependent nuclear receptor	1	1.75%
phosphatase	1	1.75%
transcription regulator	13	22.81%
transporter	2	3.51%
other	27	47.37%

Plasma Membrane	N	%
enzyme	14	18.42%
G-protein coupled receptor	3	3.95%
ion channel	5	6.58%
kinase	2	2.63%
peptidase	1	1.32%
transmembrane receptor	6	7.89%
transporter	11	14.47%
other	34	44.74%

Cytoplasm	N	%
enzyme	182	49.86%
ion channel	2	0.55%
kinase	11	3.01%
peptidase	20	5.48%
phosphatase	5	1.37%
transcription regulator	9	2.47%
transporter	30	8.22%
other	106	29.04%

Extracellular space	N	%
cytokine	1	1.01%
enzyme	7	7.07%
growth factor	2	2.02%
peptidase	4	4.04%
transporter	15	15.15%
other	70	70.71%

Unknown location	N	%
enzyme	3	7.50%
kinase	2	5.00%
other	35	87.50%

Figure 3.1 Types and cellular location of proteins identified from bovine adipose tissue.

Mature adipocytes represents the main cellular type in adipose tissue, but other cell types including macrophages, endothelial cells, preadipocytes and stem cells may also be present in adipose tissue.

3.3.2 Functional proteome of bovine adipose tissue

The functional analysis identified the most relevant biological functions of the identified proteins dataset. Proteins were associated with biological functions according to the Ingenuity Knowledge Base (IPA®) and a right-tailed Fisher's exact test calculated a p-value determining the probability that each function assigned to that dataset is due to chance alone. Twenty-six biological functions of proteins at molecular and cellular levels were ($p<0.05$ or $-\log(\text{p-value})>1.3$)

identified in bovine adipose tissue. Lipid metabolism, small molecule biochemistry, cell death and survival, cellular function and maintenance, and cellular assembly and organization were the five most relevant predicted functions (Figure 3.2).

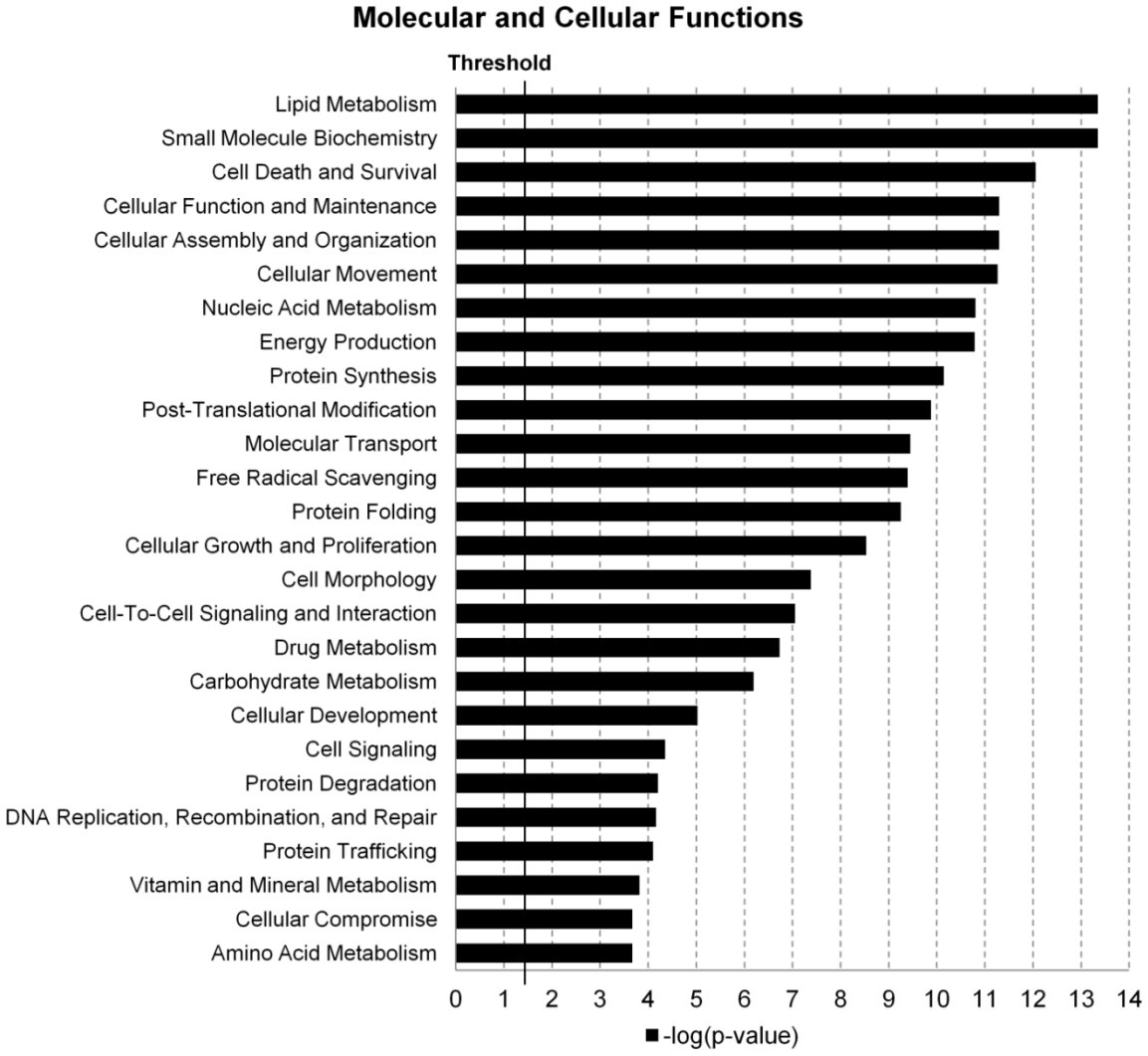


Figure 3.2 Molecular and cellular functions in bovine adipose tissue.
The likelihood of the association between the proteins in the dataset and a biological function is represented as –log(p-value), with larger bars being more significant than shorter bars. The vertical line indicates the cutoff for significance (p-value of 0.05).

3.3.3 Protein expression changes to adipose depot and diet

Fat depot and diet influenced protein expression; however a two-factor ANOVA analysis revealed that there was minimal interaction between diet and fat depot on protein expression. The expression of 51.0% of proteins was affected by fat depot and 46.9% was not affected by either diet or depot (Table 3.1). Between subcutaneous and visceral adipose tissues, 57.2% of proteins exhibited less than a 1.5 fold difference (FD) ($-1.5 < FD < +1.5$) in expression, while 42.8% varied more than 1.5 FC ($-1.5 \leq FD \geq +1.5$) in steers fed the control diet (Figure 3.3A). In steers fed the high fat diet, 51.9% of proteins varied less than 1.5 FD ($-1.5 < FD < +1.5$) in expression while 48.1% exhibited expression above this level ($-1.5 \leq FD \geq +1.5$) (Figure 3.3B). When the control and high fat diet were compared, most proteins (87.1%) changed less than 1.5 fold change (FC) ($-1.5 < FC < +1.5$) in subcutaneous adipose tissue (Figure 3.3C), while 93.4% of proteins exhibited less than 1.5 FC ($-1.5 < FC < +1.5$) in visceral fat tissue (Figure 3.3D). Expression values for all proteins according to groups are shown in supplementary Table S3.1.

Table 3.1 Effects of fat depot, diet and fat depot x diet interaction on protein expression

% Proteins	# Proteins	Fat depot	Diet	Interaction FxD
47.80%	326	<0.01	NS	NS
46.92%	320	NS	NS	NS
3.08%	21	<0.01	<0.01	NS
1.91%	13	NS	<0.01	NS
0.15%	1	<0.01	<0.01	<0.01
0.15%	1	NS	<0.01	<0.01

Fat depot, diet and their interaction have a significant effect on protein expression when a p-value <0.01 is assigned, while NS indicates no significant effect ($p>0.01$). P-values were calculated for Two-Way ANOVA analysis.

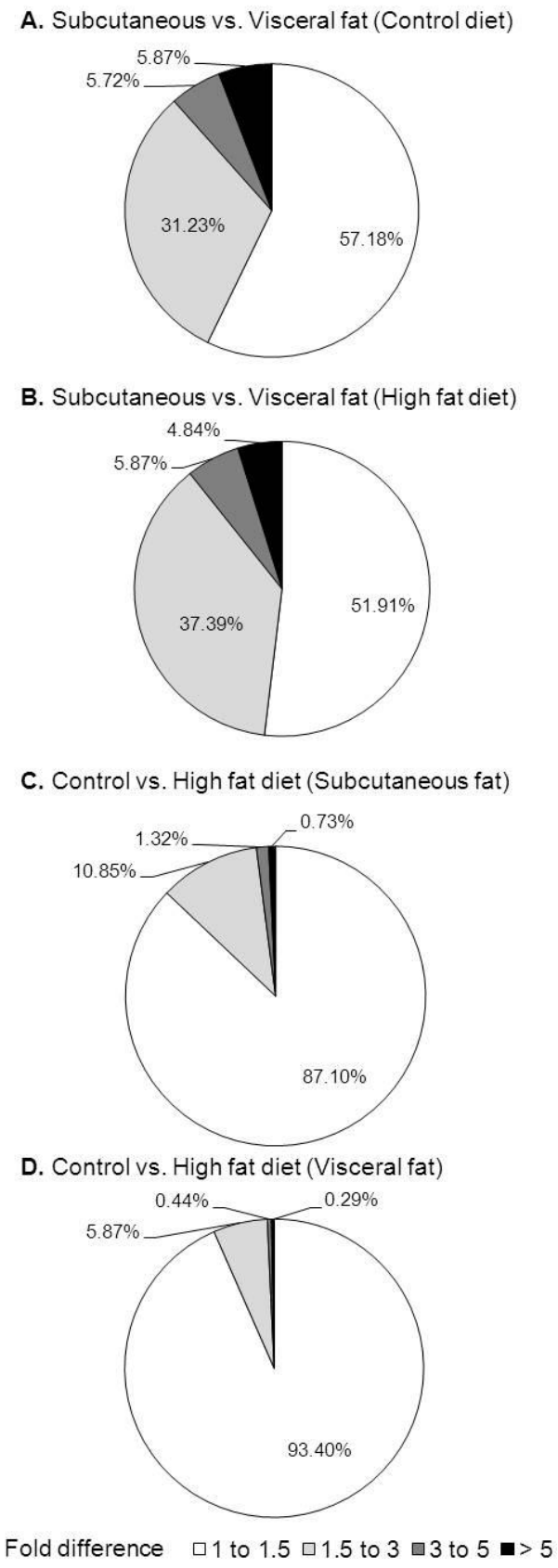


Figure 3.3 Proteome expression changes due to fat depot and diet.

Changes in expression of 682 proteins are represented by fold change differences (1 to 1.5, 1.5 to 3, 3 to 5, and >5) in adipose tissue collected from different depots in steers fed control of high fat diets: A (Subcutaneous vs. visceral adipose tissue in steers fed Control diet), B (Subcutaneous vs. visceral adipose tissue in steers fed high fat diet), C (Control vs. high fat fed steers using subcutaneous fat tissue), and D (Control vs. high fat fed steers using visceral adipose tissue).

3.3.4 Functional analysis of proteins differentially expressed by fat depot or diet

Functional analysis of differentially expressed (DE) proteins ($-1.5 \leq FC \geq +1.5$ and p-value<0.05) was undertaken according to the fat depot and diet fed. In total 252 DE proteins were identified between C.SAT and C.VAT, 270 between HF.SAT and HF.VAT, 39 between C.SAT and HF.SAT, and 15 between C.VAT and HF.VAT (Table 3.2). The top 5 molecular and cellular functions of DE protein sets from the above comparisons are listed in Table 3.2. Comparison of DE proteins between subcutaneous and visceral adipose tissues, irrespective of the diet, showed lipid metabolism as one of the most relevant functions. The Downstream Effects Analysis (IPA®) revealed that the visceral adipose tissue of steers fed control diet exhibited an increase in the activity of oxidation of lipids, oxidation of fatty acids and synthesis of lipids as compared to subcutaneous adipose tissue (z-score > 2). Similarly, steers fed the high-fat diet exhibited increased activity of lipid synthesis and the efflux of cholesterol in visceral fat than in subcutaneous fat. Furthermore, the number of upregulated proteins involved in lipid metabolism in subcutaneous fat was less than half of that in visceral fat with both diets. A total of 36 proteins were affected by diet (p<0.01). Among these, 6 proteins are known to participate in lipid metabolism and expression of three were notably higher in steers fed high fat diets: stearoyl-CoA desaturase (delta-9-desaturase) (SCD), apolipoprotein C-III (APOC3), and annexin A6 (ANXA6). While perilipin 1 (PLIN1), malate dehydrogenase 1, NAD (soluble) (MDH1), and integrin, alpha 6 (ITGA6) were expressed at higher levels

in steers fed the control diet (Table S3.1). Due to the limited number of DE proteins between control and high-fat diets, the downstream effects analysis was not able to predict activation states (increased or decreased) for biological functions influenced by diet in these datasets.

Table 3.2 Top 5 categories of molecular and cellular functions of differentially expressed proteins according to adipose tissue depot and diet

Comparison	Functional Category	Functions involved	p-value range	DE Proteins	Up-regulated	Down-regulated
C.SAT / C.VAT (n=252)	Energy Production	11	$1.48x10^{-9}$ - $2.21x10^{-3}$	34	7	27
	Lipid Metabolism	43	$1.48x10^{-9}$ - $4.23x10^{-3}$	51	16	35
	Small Molecule Biochemistry	62	$1.48x10^{-9}$ - $4.78x10^{-3}$	72	19	53
	Post-Translational Modification	7	$5.17x10^{-9}$ - $4.06x10^{-3}$	19	5	14
	Cellular Assembly and Organization	22	$2.12x10^{-8}$ - $6.22x10^{-3}$	55	30	25
HF.SAT / HF.VAT (n=270)	Cell Morphology	14	$3.20x10^{-12}$ - $5.64x10^{-3}$	42	18	24
	Cellular Assembly and Organization	22	$3.20x10^{-12}$ - $5.88x10^{-3}$	65	34	31
	Protein Synthesis	7	$1.12x10^{-10}$ - $4.62x10^{-3}$	38	9	29
	Cellular Function and Maintenance	17	$2.02x10^{-9}$ - $5.43x10^{-3}$	87	41	46
	Lipid Metabolism	48	$6.97x10^{-9}$ - $6.36x10^{-3}$	60	16	44
C.SAT / HF.SAT (n=39)	Cell Morphology	10	$3.03x10^{-4}$ - $3.76x10^{-2}$	5	1	4
	Lipid Metabolism	29	$9.28x10^{-4}$ - $4.59x10^{-2}$	5	1	4
	Small Molecule Biochemistry	40	$9.28x10^{-4}$ - $4.59x10^{-2}$	12	2	10
	Carbohydrate Metabolism	7	$1.74x10^{-3}$ - $1.04x10^{-2}$	3	2	1
	Cell-To-Cell Signaling and Interaction	24	$1.74x10^{-3}$ - $4.76x10^{-2}$	12	3	9
C.VAT / HF.VAT (n=15)	Cellular Development	26	$4.41x10^{-5}$ - $4.70x10^{-2}$	8	3	5
	Cellular Growth and Proliferation	19	$4.41x10^{-5}$ - $2.36x10^{-2}$	9	3	6
	Cellular Movement	27	$2.49x10^{-4}$ - $4.29x10^{-2}$	6	2	4
	Cellular Assembly and Organization	44	$4.84x10^{-4}$ - $4.70x10^{-2}$	8	3	5
	Cellular Function and Maintenance	25	$4.84x10^{-4}$ - $4.70x10^{-2}$	8	2	6

C.SAT: Control diet/ Subcutaneous adipose tissue, C.VAT: Control diet/Visceral adipose tissue, HF.SAT: High fat diet/Subcutaneous adipose tissue, and HF.VAT: High fat diet/Visceral adipose tissue. Functions involved: number of detected molecular and cellular functions that are involved with the main functional category. P-value range: presents the range of p-values from lowest to highest of functions for each functional category, being $p<0.05$ significant. DE proteins: amount of differentially expressed proteins between group comparisons that participate in respective function.

3.3.5 Relationship between protein expression and fat traits

Subcutaneous fat thickness and adipocyte size are two measures of adiposity with thickness ranging from 11 to 27 mm, and adipocyte size ranging from 118 to 163µm among steers in the current study. Pearson correlations between these adiposity traits and expression of proteins identified the top 10 significant ($p<0.05$) correlations (Tables 3.3 and 3.4). Three out of the top ten proteins correlated with subcutaneous fat thickness are involved in lipid metabolism (DBI, FABP5 and NQO1), while only one of the top ten proteins correlated to adipocyte size is involved with lipid metabolism (FABP4). Subcutaneous fat thickness and adipocyte size were positively correlated ($r=0.377$), however, all the top 10 proteins that correlated with each of these respective traits were different.

Table 3.3 Top 10 positive correlations between protein expression and thickness of subcutaneous fat

Protein ID	Gene ID	Description	R	p-value
Q3SYV4	CAP1	adenylate cyclase-associated protein 1	0.628	0.009
F1MHB8	QPRT	quinolinate phosphoribosyltransferase	0.626	0.009
P07107	DBI	diazepam binding inhibitor	0.611	0.012
Q3ZBH2	NQO1	NAD(P)H dehydrogenase, quinone 1	0.578	0.019
P55052	FABP5	fatty acid binding protein 5	0.577	0.019
E1BEL7	HSPB1	heat shock 27kDa protein 1	0.575	0.020
F1MNT4	LAMB1	laminin, beta 1	0.574	0.020
A8E641	DPYSL5	dihydropyrimidinase-like 5	0.557	0.025
Q3ZBD7	GPI	glucose-6-phosphate isomerase	0.554	0.026
Q32KL2	PSMB5	proteasome (prosome, macropain) subunit, beta type, 5	0.553	0.026

Correlation is significant at $p<0.05$

Table 3.4 Top 10 positive correlations between protein expression and adipocyte size

Proteins ID	Gene ID	Description	R	p-value
F1MBU7	CDCA7L	cell division cycle associated 7-like	0.673	0.004
E1B8H0	PEAK1	NKF3 kinase family member	0.594	0.015
A6QR11	NELL2	NEL-like 2 (chicken)	0.581	0.018
P11181	DBT	dihydrolipoamide branched chain transacylase E2	0.559	0.025
P00129	UQCRB	ubiquinol-cytochrome c reductase binding protein	0.544	0.029
F1MHQ4	FABP4	fatty acid binding protein 4, adipocyte	0.541	0.031
G5E5C8	TALDO1	transaldolase 1	0.526	0.037
E1BN43	ANKRD28	ankyrin repeat domain 28	0.522	0.038
F1MDH3	TLN1	talin 1	0.513	0.042
Q17QZ6	SDPR	serum deprivation response	0.510	0.044

Correlation is significant at $p<0.05$

3.3.6 Transcriptional regulation of adipose tissue

The Upstream Regulator Analysis (IPA®) revealed that 131 transcription factors (TFs) may regulate the transcription of genes that resulted in the translation of proteins that were differentially expressed between subcutaneous and visceral adipose tissue depots (n=252) in steers fed the control diet. Similarly, 145 TFs may be associated with differential regulation of proteins expressed in adipose tissues (n=270) depots from steers fed the high-fat diet. Nine TFs relevant ($p<0.05$) for each DE protein dataset had their activation state (increased or decreased) significantly predicted (z-score >2 or <-2) based on the expression of the DE proteins they regulate (Table 3.5). Most of these TFs (8 out of 9) were in common for steers fed both diets and had the same predicted activation state. Only 1 out of 9 TFs was predicted as being increased in subcutaneous fat whereas 8 were predicted to exhibit increased expression in visceral adipose tissue.

A total of 23 TFs were predicted ($p<0.05$) to regulate 39 proteins differentially expressed between diets in subcutaneous fat, while 63 TFs were predicted ($p<0.05$) to control 15 proteins differentially expressed between diets in visceral fat. However, due to the limited number of DE proteins by diet none of these transcription factors obtained predictions for their activation state.

Table 3.5 Predicted transcription factors regulating DE proteins between adipose tissue depots

		C.SAT x C.VAT				HF.SAT x HF.VAT			
Transcription factors	Description	State C.VAT	z-score	Overlap p-value	Target proteins	State HF. VAT	z-score	Overlap p-value	Target proteins
GATA4	GATA binding protein 4	↓	2.236	$4.85x10^{-4}$	6	↓	2.433	$1.39x10^{-4}$	7
KLF15	Kruppel-like factor 15	-	-	-	-	↑	2.017	$1.12x10^{-5}$	6
MEF2C	myocyte enhancer factor 2C	↑	2.433	$8.40x10^{-5}$	6	↑	2.433	$1.70x10^{-4}$	6
NFKB1	NFKB1 nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	↑	2.000	$3.49x10^{-2}$	6	↑	2.000	$6.57x10^{-2}$	8
NRIP1	nuclear receptor interacting protein 1	↓	-2.236	$5.34x10^{-4}$	5	↓	-2.646	$8.32x10^{-7}$	8
PPARA	peroxisome proliferator-activated receptor alpha	↑	2.049	$1.92x10^{-15}$	29	↑	2.692	$8.33x10^{-15}$	30
PPARG	peroxisome proliferator-activated receptor gamma	↑	3.082	$6.18x10^{-16}$	29	↑	3.397	$1.89x10^{-14}$	29
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	↑	2.739	$1.47x10^{-11}$	16	↑	3.297	$5.77x10^{-15}$	20
SPDEF	SAM pointed domain containing ets transcription factor	↑	2.236	$1.10x10^{-3}$	5	-	-	-	-
TBX5	T-box 5	↑	2.236	$6.49x10^{-5}$	5	↑	2.236	$1.19x10^{-4}$	5

C.SAT= Control diet/ Subcutaneous adipose tissue, C.VAT= Control diet/Visceral adipose tissue, HF.SAT= High fat diet/Subcutaneous adipose tissue, and HF.VAT= High fat diet/Visceral adipose tissue. State ↑ indicates increased activation of the transcription factor in C.VAT or HF.VAT and ↓ indicates decreased activation status. Z-score: indicates the level of confidence to which a transcription factor has its activation status predicted as increased or decreased, being z-score >2 or <-2 significant. Overlap p-value: analyze whether there is a statistically significant overlap between the dataset proteins and the genes that are regulated by a transcription factor with $p<0.05$ being significant. Target proteins: indicates the amount of proteins in the experiment dataset that may be regulated by the transcription factor.

3.3.7 Translation: messenger RNA to protein expression

Nine genes Acyl-CoA oxidase 2, branched chain (ACOX2), aminolevulinate dehydratase (ALAD), ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1 (ATP5C1), ELOVL fatty acid elongase 6 (ELOVL6), fatty acid binding protein 4, adipocyte (FABP4), fatty acid synthase (FASN), fibrillin 1 (FBN1), glycerol-3-phosphate dehydrogenase 1 (soluble) (GPD1), thyroid hormone responsive (THRSP) were selected to evaluate their mRNA abundance using qRT-PCR. Except for ALAD and FBN1, all genes were involved in lipid metabolism. Genes varied considerably in their expression with protein levels varying at least 3 fold in adipose tissue samples. Measures of mRNA and protein levels obtained from the same samples were compared in order to assess the translation output from mRNAs to their respective protein levels. Overall the correlation between mRNA and protein translation was moderate ($r=0.245$) (Table 3.6) for samples of animals at slaughter. The average correlation between mRNA and protein varied widely, with GPD1 (0.841) exhibiting the highest ($p<0.001$) correlation, while the correlation between transcription and translation for FASN (-0.037) was poor ($p>0.05$).

Table 3.6 Correlation between presence of mRNAs and their respective proteins

Gene ID	Description	R	p-value
ACOX2	acyl-CoA oxidase 2, branched chain	-0.371	0.052
ALAD	aminolevulinate dehydratase	0.764	<0.001
ATP5C1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	0.178	0.364
ELOVL6	ELOVL fatty acid elongase 6	0.395	0.038
FABP4	fatty acid binding protein 4, adipocyte	0.233	0.232
FASN	fatty acid synthase	-0.037	0.850
FBN1	fibrillin 1	-0.240	0.219
GPD1	glycerol-3-phosphate dehydrogenase 1 (soluble)	0.841	<0.001
THRSP	thyroid hormone responsive	0.444	0.018

Correlation is significant at $p<0.05$

3.3.8 Post-transcriptional regulation of protein expression in bovine adipose tissues

miRNA-protein dataset integration was attempted in order to investigate how miRNAs might play a role in the regulation of lipid metabolism in bovine adipose tissue (Figure 3.4). TargetScan was used to predict miRNAs that bind to the 3'UTR of mRNAs associated to a subset of proteins identified in this study (n=38), corresponding to the proteins involved in lipid metabolism and differentially expressed between fat depots in steers fed both diets. A total of 487 miRNA families were predicted to regulate these genes. These miRNAs were compared to another set of miRNAs (135 miRNA families) obtained according to the following method. A total of 244 microRNAs from the same adipose tissues obtained from our previous microRNA microarray analysis (Romao et al., 2012)

were filtered based on their frequency in the sampled steer population (cutoff ≥ 50% of animals) resulting in a total of 175 miRNAs. These miRNAs were grouped into families (miRNAs that share the same seed sequence), resulting in 135 miRNA families. (TargetScan www.targetscan.org/cgi-bin/targetscan/mirna_families.cgi?db=vert_61).

The comparison of the first dataset (487 miRNAs families) with the second (135 miRNA families) resulted in 119 miRNA families in common classified into different conservation status: highly conserved (conserved across most vertebrates), conserved (conserved across most mammals) and poorly conserved (conserved not beyond placental mammals). These miRNA families were found to have at least one representative expressed in bovine adipose tissue (Romao et al., 2012). Furthermore, they were predicted to regulate the mRNA of 38 genes involved in lipid metabolism that were found to code for DE proteins associated with subcutaneous and visceral adipose depots. A total of 8 miRNAs out of 119 miRNAs families were predicted to target more than 20% of the 38 genes transcripts (Figure 3.5). Table S3.2 lists the 38 genes involved in lipid metabolism genes with the respective miRNAs predicted to target them and detected in bovine adipose tissue, and Table S3.3 lists shows the conservation status for the 119 bovine miRNA families and the number of predicted targets for each of them.

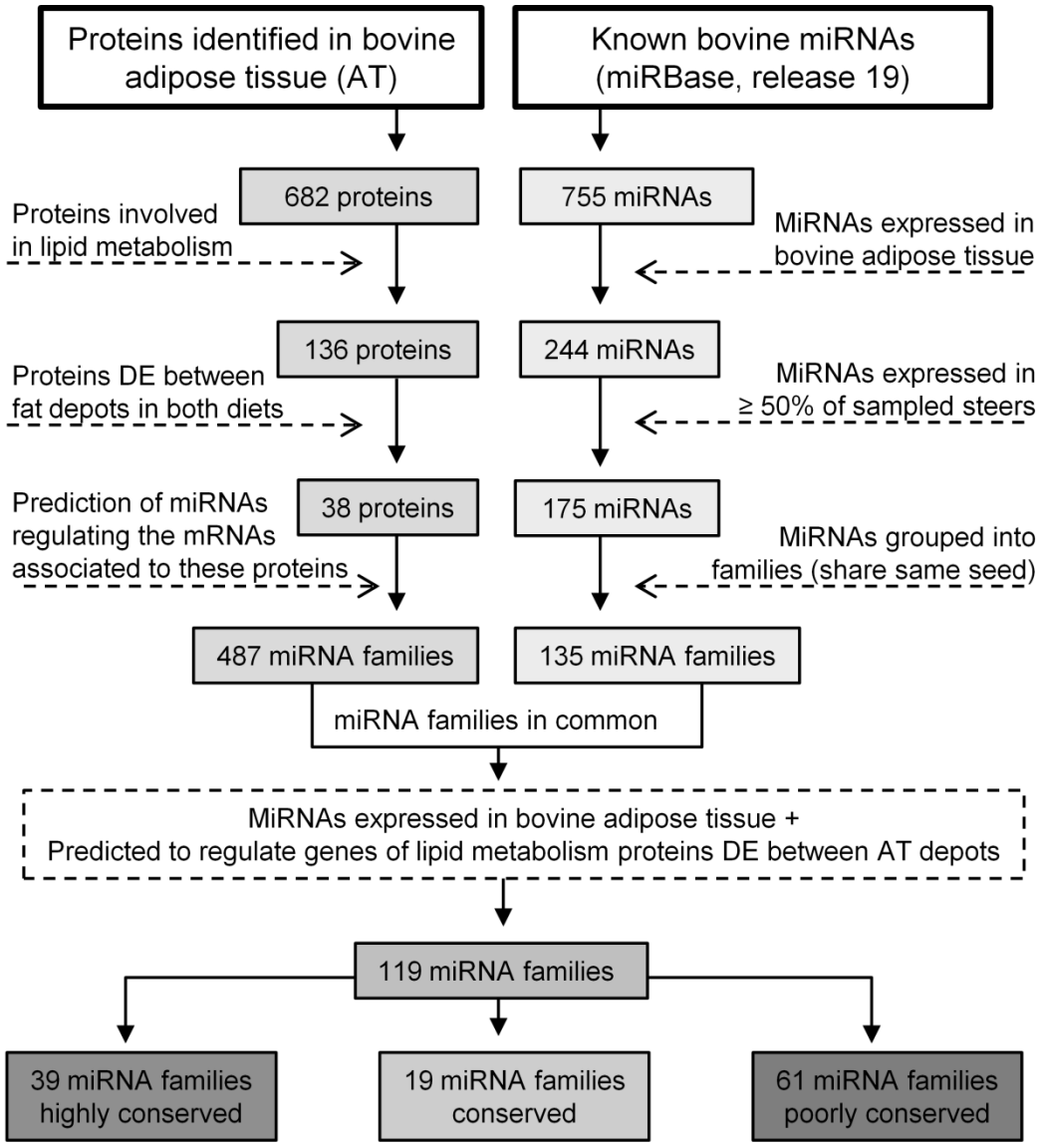


Figure 3.4 Integration of bovine miRNAs with proteins identified.
The arrows with dotted lines represent the analysis performed to filter the miRNA and protein datasets. miRNA families highly conserved refer to miRNAs conserved across most vertebrates, miRNA families conserved are conserved across most mammals and miRNA families poorly conserved are not conserved beyond placental mammals.

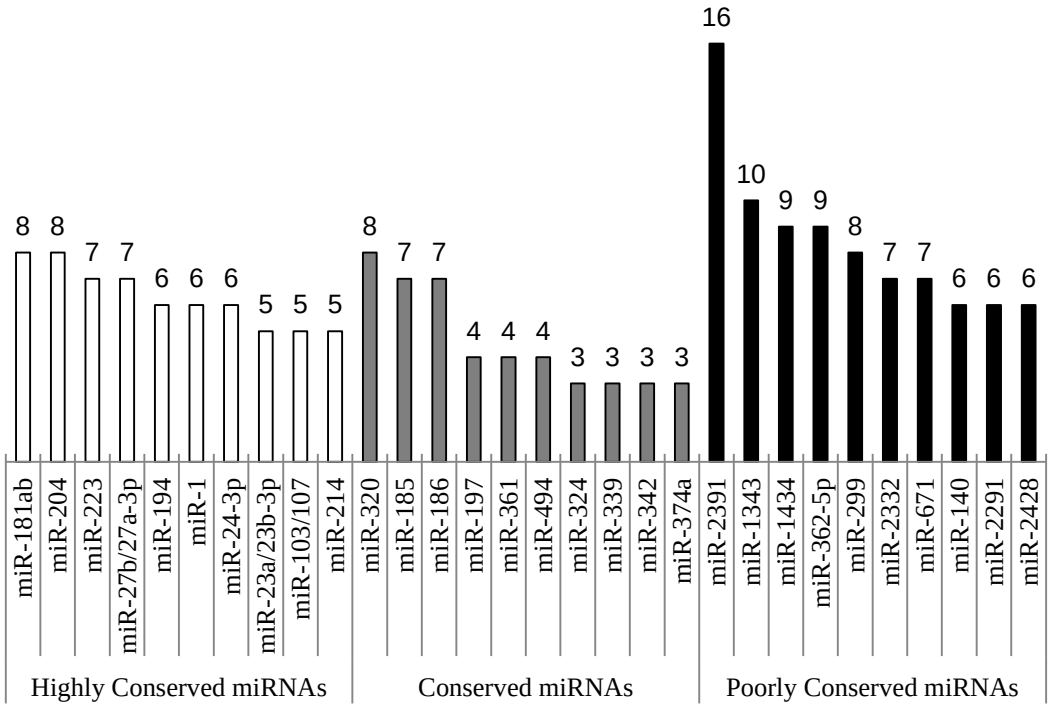


Figure 3.5 Top 10 miRNAs by conservation category predicted to regulate DE proteins between adipose depots involved in lipid metabolism.

The columns indicate the number of proteins each miRNA is predicted to regulate. miRNA families highly conserved refer to miRNAs conserved across most vertebrates, miRNA families conserved are conserved across most mammals and miRNA families poorly conserved are not conserved beyond placental mammals.

3.4 DISCUSSION

Adipose tissue is dynamic and known to play an important role in energy homeostasis. Different fat depots are known to display distinct metabolic characteristics, such as distinctive gene expression profiles, and thus likely have distinctive physiology (Hishikawa et al., 2005; Bjørndal et al., 2011).

Additionally, the function and regulation of adipose tissue may be affected by many other factors, such as diet (Zhao et al., 2010a), age (Zhang et al., 2011) and stress (Hosogai et al., 2007). Previous studies attempted to characterize the molecular regulation of bovine adipose tissue at the transcript level under different dietary regimens (Joseph et al., 2010), adiposity conditions (Taniguchi et al., 2008a) and between breeds of cattle (Jin et al., 2012). However, it is known that mRNA expression profiles may not provide the best representation of phenotype, considering that correlations between mRNA and protein expressions are often low (Gry et al., 2009; Ghazalpour et al., 2011; Schwanhausser et al., 2011). Therefore to the best of our knowledge, this study is the first to examine the molecular biology of bovine adipose tissue (*in vivo*) using a high throughput proteomic based approach.

In this study, 682 proteins differing in their cellular locations were characterized and quantified (Figure 3.1) demonstrating that our extraction procedure was capable of retrieving proteins from the main cellular compartments of adipose tissue. The diversity of protein types detected is an indication that the protein extraction was able to capture a balanced portion of the adipose tissue proteome. However, 682 proteins represent only a fraction of the total proteins expressed in bovine adipose tissue as 18,034 genes were previously reported to be expressed in the subcutaneous fat of beef cattle (Jin et al., 2012). Future studies to improve the protein identification will provide vital information of protein expression in bovine adipose tissue. In addition, mature adipocytes are the main cell type in adipose tissue, however it is important to take into account that

adipose tissue is consists of different cells, including preadipocytes, macrophages, endothelial cells and stem cells. Therefore the results from this study do not refer exclusively to adipocytes, but to the biology of adipose tissue as a whole.

Not surprisingly, the IPA core analysis predicted lipid metabolism as the major function of bovine adipose tissue, but proteins involved in many other functions (Figure 3.2) were also identified, supporting the role of adipose tissue in other metabolic activities such as an endocrine functions (Galic et al., 2010; Poulos et al., 2010) as supported by the production of adiponectin. This adipokine, detected in our study (Table S3.1), is known as an important regulator of glucose and fatty acid metabolism in skeletal muscle (Sweeney, 2011). Immune function proteins were represented as well (Table S3.1), for example with the presence of complement component 3 protein (C3), which is part of the immune system complement pathway (Mayilyan, 2012).

All 682 proteins detected in this study were present in both subcutaneous and visceral adipose tissue from steers, regardless of diet. The presence of the same proteins in all groups suggests that they are involved in core functions in terms of the biological processes as predicted by their functional analysis (Figure 3.2). However, the activity status of these processes may be different among the groups as fat depot has a significant impact on the expression of most proteins and diet also affects protein expression to a lesser extent (Table 3.1 and Figure 3.3). More DE proteins were observed between adipose depots than between two diets suggesting that the molecular mechanism of adipogenesis differs between these depots. Downstream analysis supports this contention as demonstrated by the

upregulated function of lipid synthesis and lipid oxidation in visceral fat tissue. Previous studies using bovine adipocyte culture (Rajesh et al., 2010) and human adipose tissue (Pérez-Pérez et al., 2009) together with our findings support that subcutaneous and visceral adipose tissue have differentially expressed proteins and are physiologically distinct (Baglioni et al., 2012).

Differentially expressed proteins between fat depots were involved with lipid metabolism, energy production and small molecule biochemistry of diet (Table 3.2). Therefore, it can be inferred that bovine subcutaneous and visceral fat differ in their lipid metabolism capabilities. Our results showing that lipid oxidation and synthesis of lipid functions are predicted to be significantly increased in visceral fat in control steers and lipid synthesis in steers fed the high fat diet (z-score > 2), concur that visceral adipose tissue is known to be more metabolically active than subcutaneous adipose tissue (Pérez-Pérez et al., 2009) and suggesting that visceral fat is more involved in energy homeostasis while subcutaneous might be more involved with energy storage.

However, dietary fat content also influenced the translation of a number of proteins despite having a lower impact on adipose tissue regulation. Its effect was more pronounced on subcutaneous adipose tissue as compared to visceral adipose suggesting that depots respond differently to the inclusion of fat in the diet. This finding may have implications to beef quality, as visceral fat is discarded, while subcutaneous fat is partially consumed with the muscles and seems to be more responsive to dietary fat. For instance, stearoyl-CoA desaturase (SCD) was more highly expressed in steers fed the high fat diet (flax supplemented). This enzyme

converts saturated fatty acids into monounsaturated fatty acids such as stearic acid into oleic acid, which is the most abundant fatty acid in bovine adipose tissue (Smith et al., 2006). SCD is important to bovine preadipocyte differentiation and its product (oleic acid) softens fat, a property that can impact beef quality (Smith et al., 2006), these findings suggest that altered SCD protein level may be an indicator of the beef quality and in the future this protein targeted nutritional management may improve the healthy fat component in beef product. A recent study on fatty acid profiles of the same bovine subcutaneous fat also revealed that flax seed supplementation increased alpha linoleic acid content (He et al., 2012b).

Interestingly, steers fed the high fat diet had a down regulation of integrin alpha 6 (ITGA6), which was found to induce growth arrest in preadipocytes (Liu et al., 2005), which favours differentiation over proliferation. Therefore, both adipose tissues in steers fed the high fat diet may have been undergoing a more intense preadipocyte proliferation. Our previous study using the same steers showed that those fed the high fat diet expressed more miRNAs in adipose tissue than those fed the control diet (Romao et al., 2012), indicating that the higher fat content of the diet may increase the regulatory role of miRNAs in adipose tissue metabolism. There were fewer DE proteins between diets, yet these proteins are involved in important functions such as cellular development, cell morphology and lipid metabolism. A previous study (Joseph et al., 2010) showed that dietary manipulation of fat content in bovine diets can impact the expression of several lipogenic genes, but our results did not find an extensive impact at the protein level.

Several proteins in this study had significant positive correlation with adiposity traits such as, subcutaneous fat thickness and adipocyte size (Tables 3.3 & 3.4). The fatty acid binding protein 5 (FABP5) and FABP4 were positively correlated with subcutaneous fat thickness and adipocyte size, respectively. FABP4 and FABP5 are associated with the terminal differentiation of adipocytes and are responsible for the intracellular transport of fatty acids (Samulin et al., 2008; Cristancho and Lazar, 2011). These findings suggest that an increased expression of these proteins supports an increased degree of adiposity in this fat depot. NAD(P)H dehydrogenase quinone 1 (NQO1) was found to be the best marker for the differentiation process of swine adipocytes *in vitro*, being induced during adipogenesis (Monaco et al., 2012) and in our study it was positively correlated with subcutaneous fat thickness. This suggests that this protein is also associated with bovine adipogenesis although previous studies have revealed that molecular mechanisms of bovine adipocyte differentiation is different than porcine adipogenesis (Taniguchi et al., 2008b). Transaldolase 1 protein (TALDO1) was also significantly correlated to adipocyte size. Interestingly, this protein was upregulated by a 2 fold magnitude in a study comparing subcutaneous fat of steers (castrated) and bulls (not castrated) (Zhang et al., 2012). The differential expression of this protein suggests that it might be involved in the heightened deposition of subcutaneous fat in steers as compared to bulls. Other proteins were significantly correlated with fat traits, but their function as related to adipogenesis is unknown.

Proteins are the final stage of gene expression in adipose tissue. Adipocyte protein levels may be regulated by several upstream mechanisms, including transcriptional regulatory factors (Rosen and MacDougald, 2006), miRNAs (Romao et al., 2011), and epigenetics (Fujiki et al., 2009). The upstream regulator analysis performed in this study predicted transcription factors that might possibly be involved in bovine adipose tissue regulation.

Transcription factors play a fundamental role in the regulation of gene expression, impacting the protein output. Furthermore, they may account for a considerable portion of the genes and expressed proteins as illustrated by the finding in the human genome where they account for at least 6% of total protein coding genes (Vaquerizas et al., 2009). It is important to note that from the total proteins profiled in our study only two were transcription factors (~ 0.3% of total). It is known that transcription factors are expressed at much lower levels than non-transcription factor genes/proteins (Vaquerizas et al., 2009). Therefore, transcription factors are much more difficult to quantify in comparison to proteins which are expressed at increased levels. Therefore, this bioinformatics analysis provided important information about the expression of adipose transcription factors regulating DE proteins between subcutaneous and visceral adipose depots, which would not be obtained with LC-MS/MS alone.

Except for myocyte enhancer factor 2C (MEF2C), SAM pointed domain containing ets transcription factor (SPDEF), and T-box 5(TBX5), the functions of all other identified TFs can involve aspects of lipid metabolism or adipocyte development (Table 3.5) including peroxisome proliferator activated receptor

alpha (PPAR-α), and the adipogenesis master regulator peroxisome proliferator activated receptor gamma (PPAR-γ) (White and Stephens, 2010). Both PPAR-γ and PPAR-α can regulate more than 10% of the differentially expressed proteins between adipose depots. This shows their crucial roles to the specific regulation of each fat depot leading to the unique physiological characteristics of each adipose site.

Transcription factors regulate the levels of mRNA as they control the pace of transcription, but they do not alter the translation of proteins from mRNA. Several reports describe an overall modest correlation between mRNA and protein expression (Gry et al., 2009; Ghazalpour et al., 2011; Schwanhausser et al., 2011) and our results with a weak correlation of 0.245 are in agreement (Table 3.6). This suggests that mRNA levels may not be good predictors of protein expression for all genes and therefore not an accurate predictor of phenotype. In this context, miRNAs may be considered as one of the key post-transcriptional regulators, especially given the magnitude of miRNAs expressed in bovine adipose tissue (Jin et al., 2009; Jin et al., 2010; Romao et al., 2012). miRNAs are likely to play an important fine-tuning role in bovine adipogenesis by binding to mRNA targets and regulating their availability to translation which modulates gene expression and may reduce undesired fluctuations in proteins levels (Ebert and Sharp, 2012). Our computational prediction identified a large number of miRNAs that may contribute to the physiological differences between bovine subcutaneous and visceral fat by targeting the transcripts of DE proteins between fat depots. Among the top 10 highly conserved miRNAs (Figure 3.5), miR-

103/107 (Xie et al., 2009a; Trajkovski et al., 2011) and miR-27b/27a-3p (Karbiener et al., 2009; Kim et al., 2010) families are well known for their roles in the regulation of adipogenesis, a function that may be universal in most vertebrates. Several poorly conserved miRNAs were predicted to regulate transcripts originating DE proteins. Three bovine specific miRNAs (miR-2391, miR-1434 and miR-2332) were among the top 10 poorly conserved miRNAs (Figure 3.5), suggesting that they are potential bovine specific post regulatory mechanisms in adipogenesis. MiR-2391 was the miRNA with the highest regulatory potential over the set of genes associated with proteins involved in lipid metabolism that were differentially expressed between fat depots. It is worth considering that the status of bovine specific miRNAs in miRBase (miRBASE, 2012) may be temporary as these miRNAs might be shared among other ruminant species that have not been reported yet.

In conclusion, the results obtained from this study revealed that the profile of the bovine adipose proteome differs between fat depots, indicating important functional and physiological differences such as a higher metabolic activity of visceral fat. This finding might indicate a more pronounced role of subcutaneous fat in energy storage while visceral might be more active in lipid metabolism and energy balance. Subcutaneous fat was the most responsive to dietary fat in terms of DE proteins and this result has the potential to be explored as a strategy to improve fat quality through diet manipulation. Bovine adipogenesis is a complex biological process in which several transcription factors are predicted to regulate gene expression at transcription level. Besides, miRNAs may also play an

important role in post transcriptional regulation as protein output from translation was not consistent with mRNA levels. Several miRNAs expressed in bovine adipose tissue were predicted to regulate genes coding to DE proteins associated to fat depots, suggesting their roles in bovine adipogenesis. These results improve our understanding on adipogenesis and may help the development of feeding strategies to manipulate adiposity in beef cattle, which is an important aspect not only to meat quality and animal production but also to human health.

3.5 APPENDIX

Table S3.1. Protein annotation, normalized abundance by group and treatment effects.

http://www.plosone.org/article/fetchSingleRepresentation.action?uri=info:doi/10.1371/journal.pone.0083211.s002

Table S3.2. miRNAs that target lipid metabolism genes whose protein were differentially expressed between adipose depots in both diets.

http://www.plosone.org/article/fetchSingleRepresentation.action?uri=info:doi/10.1371/journal.pone.0083211.s003

Table S3.3. Number of predicted targets for the 119 bovine miRNA families and their conservation status.

http://www.plosone.org/article/fetchSingleRepresentation.action?uri=info:doi/10.1371/journal.pone.0083211.s004

Table S3.4. Formulation and nutritional composition of Control and High fat diets.

http://www.plosone.org/article/fetchSingleRepresentation.action?uri=info:doi/10.1371/journal.pone.0083211.s005

Table S3.5. Primer sequences

http://www.plosone.org/article/fetchSingleRepresentation.action?uri=info:doi/10.1371/journal.pone.0083211.s006

Protocol S3.1. Label Free Quantitation LCMS Methodology Report.

http://www.plosone.org/article/fetchSingleRepresentation.action?uri=info:doi/10.1371/journal.pone.0083211.s001

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4 MicroRNAs in bovine adipogenesis: genomic context, expression and function[7]

4.1 INTRODUCTION

Adipose tissue biology is fundamental to functional physiology as fat is not only the major tissue of energy storage in mammals, but also an important endocrine organ (Galic et al., 2010; Poulos et al., 2010). Dysfunctions in adipose tissue metabolism have become an important health concern as obesity has reached epidemic proportions (Catenacci et al., 2009) with approximately a quarter of the world's population being overweight or obese (Nguyen and Lau, 2012). The consumption of high fat foods is clearly associated with obesity (Hariri and Thibault, 2010) and as a consequence both breeding and nutritional programs have focused on increasing the leanness of beef (Wang and Beydoun, 2009; McNeill et al., 2012). Meat is an important protein source and consumption is predicted to increase by 3.2kg per capita until 2021 (OECD-FAO, 2012). Fat is an important component in meat quality and impacts animal productivity (Hausman et al., 2009; Dodson et al., 2010b); therefore an understanding of the molecular regulation of adipogenesis is pivotal in the development of strategies to manipulate adiposity and improve beef quality.

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Adipogenesis involves the development of mature adipocytes from preadipocytes (Large et al., 2004) and is responsible for modulating adiposity in individuals. Adipogenesis is genetically regulated and studies have shown the importance of adipogenic transcription factors (PPARγ, C/EBPs, KLFs and SERBP) in fat development as they regulate the expression of adipogenic genes involved in adipocyte differentiation (Lefterova and Lazar, 2009; White and Stephens, 2010). However, the regulatory mechanisms of microRNAs (miRNAs) in adipose tissue are not well understood.

miRNAs are small non-coding RNAs composed of approximately 22 nucleotides that repress gene expression by binding to messenger RNAs in a sequence-specific manner (He and Hannon, 2004). Studies in humans and mice have shown that miRNAs regulate adipogenesis in a pro or anti-adipogenic manner (Xie et al., 2009b; Romao et al., 2011) through repression of various genes, including transcriptional factors such as *PPARγ*, *PPARα* and *KLF5* (Lin et al., 2009; Kim et al., 2010; Kinoshita et al., 2010; Martinelli et al., 2010).

Studies using bovine adipose tissue have shown that miRNA expression profiles change according to internal and external environmental factors such as subcutaneous fat thickness (Jin et al., 2010), fat depot (subcutaneous vs. visceral fat) and dietary manipulation (high vs. low fat content) (Romao et al., 2012). However, it is not clear how miRNA expression and function are impacted by the compositional structure of DNA that surrounds miRNA genes, also known as genomic context characteristics. These might include features such as the distance between miRNA genes, which has been suggested to be a determinant for

coordinated miRNA expression (Wang et al., 2011) or the location of miRNA genes in relation to protein coding genes (intergenic, intronic, exonic, or mirtron). The elucidation of these aspects may provide important clues with regard to the regulation and function of miRNAs in bovine adipogenesis. Therefore, this study aimed to determine how genomic context of miRNA genes and their conservation are associated with the expression and function in bovine subcutaneous adipose tissue.

4.2 MATERIAL AND METHODS

4.2.1 Animal study and sample collections

A total of eight, 12 month old British-continental crossbred beef steers were housed in individual pens at the Lethbridge Research Centre. Steers were selected based on similar body weight (456 ± 20 kg) and offered either a low fat diet containing 2.7% fat, (Control group, n=4) or a high fat diet containing 7.1% fat. (High fat group, n=4) *ad libitum*. Fat content of the diet was increased by including 10% flaxseed in the diet. Steers had free access to water and diets were fed for 14 weeks until steers were slaughtered at about 15.5 months of age. Throughout the experiment several performance measures were recorded including body weight gain, feed intake and feed conversion. Carcass traits including cutability, backfat thickness and adipocyte size were also recorded and reported previously (He et al., 2012b; He et al., 2012c). Subcutaneous fat was collected from each steer through biopsy at three six-week intervals (12, 13.5 and 15 months). The first biopsy collection (0.2-0.5g) was performed at 15 cm to the

left of the last thoracic vertebrae of each steer. Subsequent biopsies were performed within the same area but 4 and 8 cm left of the scar from the initial biopsy. Subcutaneous fat (backfat) samples were immediately frozen in liquid nitrogen, and stored at -80°C until analyzed. The animal study was approved by the Animal Care Committee of Lethbridge Research Centre, Agriculture Agri-food Canada with ACC# 0930.

4.2.2 **RNA extraction**

Total RNA extraction was performed by homogenizing the frozen fat tissue samples with TRIZOL® (TRI reagent, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions for samples with high fat content. The concentration of total RNA was measured using the NANODROP® spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA, US) and RNA integrity was measured using the Agilent 2100 BIOANALYZER® (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). RNA with integrity number (RIN) >7.8 and concentration > 200ng/µl was used for miRNA microarray and qRT-PCR analysis.

4.2.3 Microarray Analysis

miRNA profiling of subcutaneous adipose tissue samples (n=24) at three different time points (0, 6 and 12 weeks) from 8 cattle (4 fed each of the two diets) was performed. AGILENT 8 × 15K miRNA array V3 (Agilent Technologies, Santa Clara, CA, USA) was customized to profile 672 bovine

miRNAs based on the miRBase (Release 15). In brief, total RNA (100 ng) was firstly labeled with the AGILENT miRNA Complete Labeling and Hyb Kit (Version 2.1) by dephosphorylation with calf intestinal phosphatase, followed by denaturing and ligation with Cyanine3-pCp at the 3' end. The labeled RNA was hybridized with array slides with hybridization buffer and 10X GE blocking agent, and incubated at 55ºC for ~20 hours. Finally, the arrays were washed with GE buffers and scanned at 5 μM resolution on an Agilent G2565CA High Resolution Scanner (Agilent Technologies). Data were processed through Agilent's Feature Extraction software version 10.7.3.1 using the protocol miRNA_107_Sep09 and the data was normalized to the 75th percentile using GeneSpring GX 11.5 (Agilent Technologies).

4.2.4 miRNA genomic context and conservation status analysis

Genomic context characteristics of the core bovine adipose miRNA genes and miRNA conservation status were obtained using online bioinformatics search tools. Bovine miRNA organization was classified as clustered or unclustered using the cluster search tool from miRBase (http://www.mirbase.org/search.shtml) considering clustered miRNAs as those with inter-miRNA distance of less than 10 kb and within the same DNA strand (Griffiths-Jones et al., 2008). miRNAs on bovine genome were classified according to their location as intronic, intergenic, exonic and mirtron by searching their genomic location in the cow genome assembly UMD3.1 using the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/) and Ensemble genome browser (http://uswest.ensembl.org/index.html). Core adipose miRNAs were

classified according to their conservation status as highly conserved, conserved or poorly conserved by searching the TargetScan 6.2 database for conservation of miRNA families (http://www.targetscan.org/cgi-bin/targetscan/mirna_families.cgi?db=vert_61) (Friedman et al., 2009).

4.2.5 Bioinformatics analysis

miRNA relevant networks were created by connecting adipose core miRNAs according to the correlation ($R^2 \geq 0.95$) of their expression over 24 samples using Relevance Networks tool (Butte et al., 2000) from the Multiple Array Viewer from Multi Experiment Viewer software (v.4.8) (Saeed et al., 2003). Prediction of target genes was performed for each core adipose miRNA using TargetScan 6.2 for mammals and customized by species (cow/Bos taurus) (http://www.targetscan.org/vert_61/). Functional analysis of genes hosting adipose core miRNAs located inside their introns was performed through IPA Core Analysis (Ingenuity® Systems, www.ingenuity.com). The same method was used for the functional analysis of miRNA predicted targets. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function was relevant to the assigned data set with significance designated at $p < 0.05$.

4.2.6 Dataset

All the microarray data in this study are in compliance with MIAME guidelines and the data have been deposited in the publicly available NCBI's Gene

Expression Omnibus Database (http://www.ncbi.nlm.nih.gov/geo/). The data are accessible through GEO Series accession number GSE50489 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50489.

4.2.7 miRNA and gene expression validation by qRT-PCR

Candidate intronic miRNAs were selected for qRT-PCR validation based on the functional analysis of their host genes. Genes associated with lipid metabolism and their intronic miRNA were selected for qRT-PCR.

miRNA expression was carried out with TAQMAN® miRNA assays according to the manufacturer's recommendation (Applied Biosystems, Foster City, CA, USA). Briefly, cDNAs were reversely transcribed from 10 ng of total RNA using 5X specific miRNA RT primer and were amplified using a 20X TAQMAN® miRNA assay. Fluorescence signal was detected with an ABI STEPONEPLUS Real-time PCR System detector® (Applied Biosystems).

In order to assess gene expression, first strand was obtained from total RNA for each sample using random primers and reverse transcription reagents (Invitrogen, Carlsbad, CA, USA) according to manufacturer's guidelines. Each PCR reaction (20µL) consisted of 2 ng of template cDNA, 2× SYBR Green I Master Mix buffer (10 µL, Applied Biosystems, Foster City, CA), and 300 n*M* forward and reverse primers. Fluorescence signal was detected with an ABI STEPONEPLUS Real-time PCR System detector® (Applied Biosystems) using the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C.

A total of 12 samples from six steers (3 from each diet) at two different ages (12 and 15 months) were used for qRT-PCR analysis with a total of 3 technical replicates per reaction for both intronic miRNA and host genes. Bta-miR-181a was selected as the reference for miRNA analysis while beta-actin was used as reference for gene expression in this study due to their uniform expression among all animals. Primers were manufactured by Invitrogen and sequences are shown in Table 4.A2. Gene expression was analyzed by relative quantification (delta delta Ct method).

4.2.8 Statistical Analysis

The means of miRNA expression and predicted targets according to miRNA conservation status (highly conserved vs. conserved vs. poorly conserved) were analysed by one-way ANOVA and compared by Tukey's test. Comparisons of means involving miRNA organization (clustered vs. non-clustered miRNAs) and miRNA location (intergenic vs. intronic miRNAs) were performed by a two tailed t-test. The miRNA expressions from qRT-PCR were tested for normality using Shapiro-Wilk test and equal variances using Levene's test, and submitted to an one-way ANOVA and the means were compared by Tukey's test. Differences were considered statistically different at $p<0.05$ and analyses were performed with SAS software v.9.0 (SAS Inst. Inc., Cary, NC).

4.3 RESULTS

4.3.1 Genomic organization of bovine miRNAs

The bovine genome is organized in 29 pairs of autosomes and 2 sex chromosomes. A total of 755 mature miRNAs have been detected in the bovine and originate from 769 miRNAs genes coded in different genomic loci of virtually every bovine chromosome, except the sex chromosome Y (miRBase, release 19). It is worth mentioning that the difference in the quantity of miRNAs and miRNA genes happens because their relationship is not one to one, as some miRNA genes may create more than one miRNA (eg. microRNA 151 gene originates miR-151-3p and miR-151-5p) and some miRNAs can originate from different miRNA genes (eg. miR-378 from miRNA 378 genes on chromosomes 4 and 7). Based on miRNA microarray analysis, approximately 30% of the known bovine miRNAs (n = 224) were detected in subcutaneous adipose tissue of which 155 miRNAs were expressed in the subcutaneous fat of all steers (n = 8) in at least one time point, being defined as the adipose tissue core miRNAs (AT core miRNAs) in this study. The genomic distribution of AT core miRNAs was highly variable as some chromosomes (chr 10, chr 23, chr 28, and chr Y) possessed no adipose core miRNA genes while others coded for several, such as chromosome X which had 18 of them (Figure 4.1). miRNA density per Mb of chromosome was also calculated for known miRNAs and AT core miRNAs in bovine genome (Figure 4.A1).

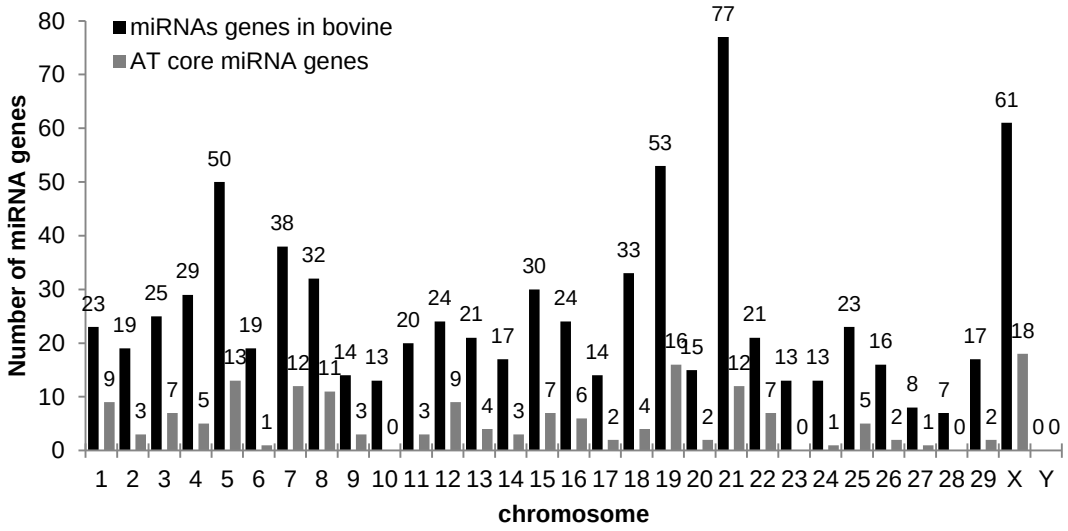

Figure 4.1 Number of miRNA genes in each chromosome.

AT core miRNAs consist of miRNAs detected in the subcutaneous adipose tissue of all steers.

Mature miRNAs (n=155) expressed in all steers were classified into three main conservation categories: 76 highly conserved miRNAs (conserved across most vertebrates), 22 conserved miRNAs (conserved across most mammals), and 57 poorly conserved miRNAs (conserved not beyond placental mammals) (TargetScan www.targetscan.org/cgi-bin/targetscan/mirna_families.cgi?db=vert_61). The genomic context of these miRNAs was also investigated in terms of organization (clustered and non-clustered) and their location as intergenic, intronic, exonic, or mirtron as shown in Figure 4.2. miRNAs in clusters represented only 26.7% of all bovine miRNAs (n=755); however, that percentage increased to 44.5% when only AT core miRNAs (n=155) were considered, which shows the importance of their role in

the core regulation of bovine adipose tissue. The organization of miRNAs in clusters was associated with conservation status as the highly conserved miRNAs were by far the most numerous, representing an overlap of 71.4% (Figure 4.2).

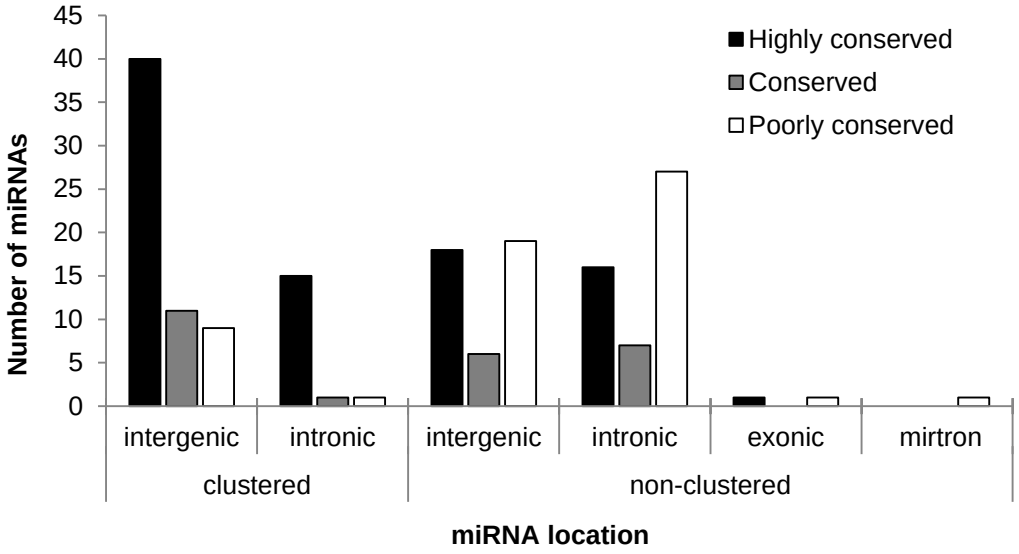

Figure 4.2 Genomic context and conservation of AT core miRNAs.

AT core miRNAs consist of miRNAs detected in the subcutaneous adipose tissue of all steers

4.3.2 miRNA expression in adipose tissue

Expression of AT core miRNAs (n=155) varied widely, as microarray intensity values (normalized) ranged from close to zero (miR-2425-3p) up to 10.9 (miR-2478). A power trendline illustrated the predictability of miRNAs behavior in terms of average expression and variation among samples (CV) with the equation $y=0.5967x^{-0.999}$, ($R^2=0.857$) where "y" represents miRNA expression and "x" is the CV (Figure 4.3). miRNA expression among steers varied considerably with type of miRNA, ranging from highly uniform such as miR-26a with a

coefficient of variation (CV) of 0.05 to highly variable such as miR-2428 with a CV of 1.90 (Figure 4.3). The average expression of miRNAs and their coefficient of variation were negatively correlated (R= -0.71) as highly expressed miRNAs tended to occur consistently among steers while poorly expressed miRNAs varied widely among individuals.

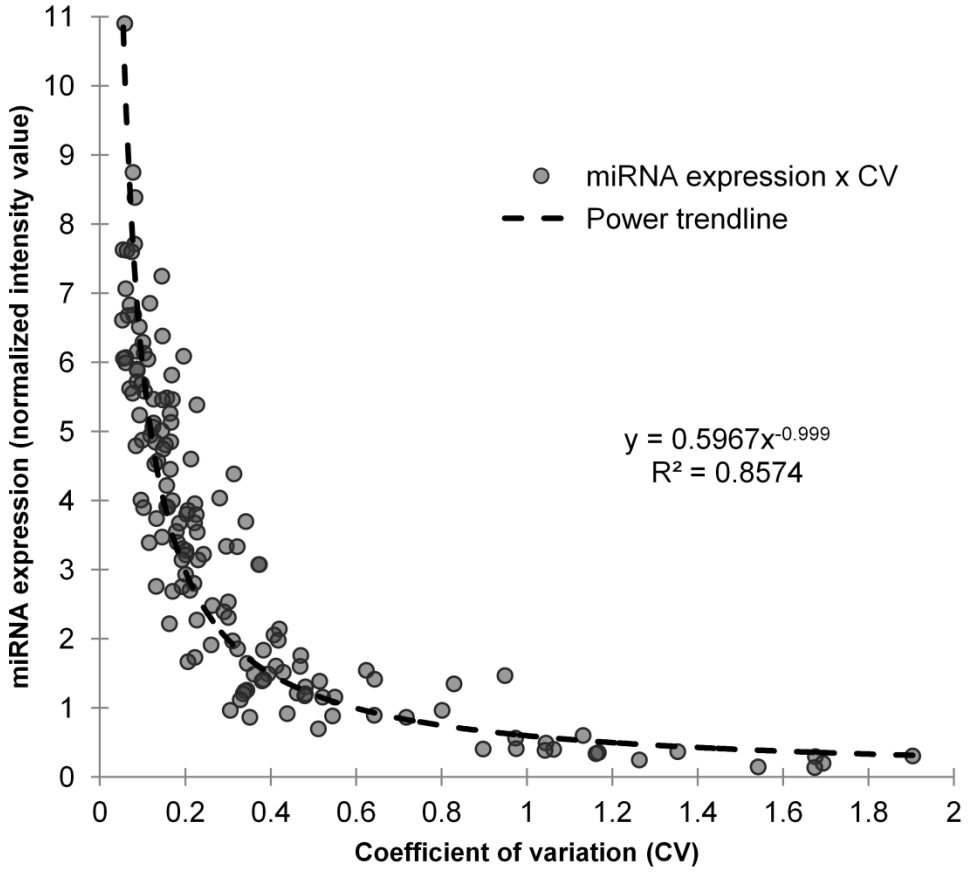

Figure 4.3 AT core miRNAs: miRNA average expression vs. coefficient of variation.

Each circle represents one unique miRNA and the coordinates of X and Y axis are the respective values for average expression of the miRNA and its coefficient of variation from different samples. AT core miRNAs consist of miRNAs detected in the subcutaneous adipose tissue of all steers.

The genomic context in which AT core miRNAs genes are located seems to be a factor impacting expression, as clustered miRNAs were more highly expressed than unclustered miRNAs (p=0.022). However, being located in an intron of a protein coding gene (intronic miRNA) or being an independent transcription unit in between genes (intergenic miRNA) did not seem to impact global miRNA levels (p=0.448). miRNAs that were highly conserved were expressed more (p<0.001) than miRNAs moderately conserved or poorly conserved (Figure 4.4).

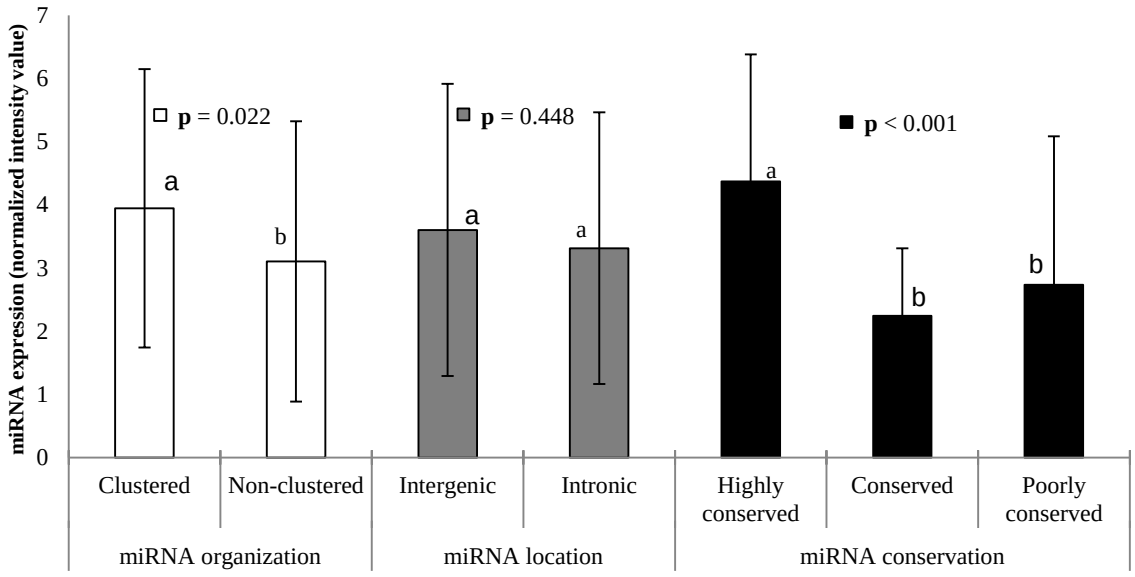

Figure 4.4 Expression of AT core miRNAs according to miRNA gene organization, location and conservation.

Columns with different letters (a,b) differ statistically within each category comparison (p<0.05). Error bars represent standard deviation. AT core miRNAs consist of miRNAs detected in the subcutaneous adipose tissue of all steers

Expression of miRNAs also varied considerably within each category according to miRNA gene locations, and with degree of conservation. For instance, miRNAs that were poorly conserved could still be highly expressed, as was observed with miR-2478 (a bovine specific miRNA) which was non-clustered and poorly conserved, but the most highly expressed miRNA in bovine adipose tissue (Table 4.A1).

4.3.3 Predicted targets of AT core miRNAs

AT core miRNAs had on average 500 predicted targets by TargetScan 6.2 (http://www.targetscan.org/), ranging from 3 target genes for miR-2892 and 1262 targets predicted for miR-30a-5p, miR-30b-5p, miR-30c, miR-30d, and miR-30f. The number of predicted targets varied ($p < 0.001$) with genomic organization and conservation, but the location of miRNAs had no ($p > 0.05$) relationship to number predicted targets (Figure 4.5). On average, highly conserved miRNAs had three times more the number of predicted targets as compared to poorly conserved miRNAs, while clustered miRNAs had 1.8 fold more predicted targets than non-clustered miRNAs.

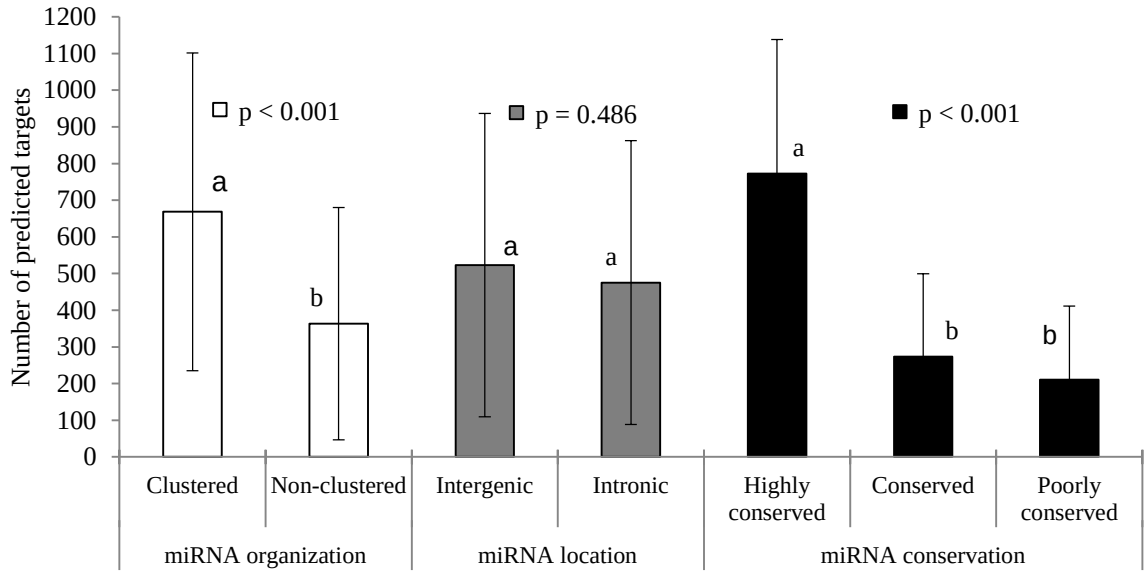

Figure 4.5 Number of predicted targets according to AT core miRNA organization, location and conservation.

Columns with different letters (a,b) differ statistically within each category comparison ($p<0.05$). Error bars represent standard deviation. AT core miRNAs consist of miRNAs detected in the subcutaneous adipose tissue of all steers.

4.3.4 Co -expression patterns of AT core miRNAs

The relationship between the expressions of AT core miRNAs across adipose tissue samples was either positively correlated, non-correlated or negatively correlated. For example, the levels of miR-20a and miR-106b were positively correlated (R = 0.99) while miR-2288 and miR-671 were negatively correlated (R = -0.76). Relevant networks analysis (Butte et al., 2000) revealed bovine miRNAs with coordinated expression that were likely to participate in common biological processes in adipose tissue. miRNA networks were generated

by examining those miRNAs that exhibited highly correlated (R^2 > 0.95) expression over the 24 adipose tissue samples (Figure 4.6). A total of six relevant networks composed of 34 miRNAs were generated, with 14 miRNAs in network 1, 12 in network 2 and 2 miRNAs in each of networks 3, 4, 5, and 6. The majority of miRNAs (70%) involved in the large networks 1 and 2 were highly conserved; while most miRNAs (75%) in small networks 3, 4, 5, and 6 were poorly conserved (Figure 4.6). Several miRNAs in the relevant networks were members of bovine miRNA clusters: miR-17-5p, 19a, 20a, 19b and 92 (cluster 17~92); miR-25 and miR-106b (cluster 106b ~ 25); miR-16b and 15b (cluster 16b~15b) and miR-15a (cluster 16a~15a) in network 1. While in network 2 let-7a and let-7b were members of the let-7a~let-7b cluster; let-7a, let-7d, and let-7f formed the let-7a~let-7d cluster and let-7f and miR-98 were members of the 98~let-7f cluster. Interestingly, let-7a and let-7f were present in more than one cluster as each of them is coded by more than one miRNA gene located on different chromosomes. miRNA pairs in networks 3, 4, 5 and 6 were highly correlated but, their miRNA genes were not organized in clusters.

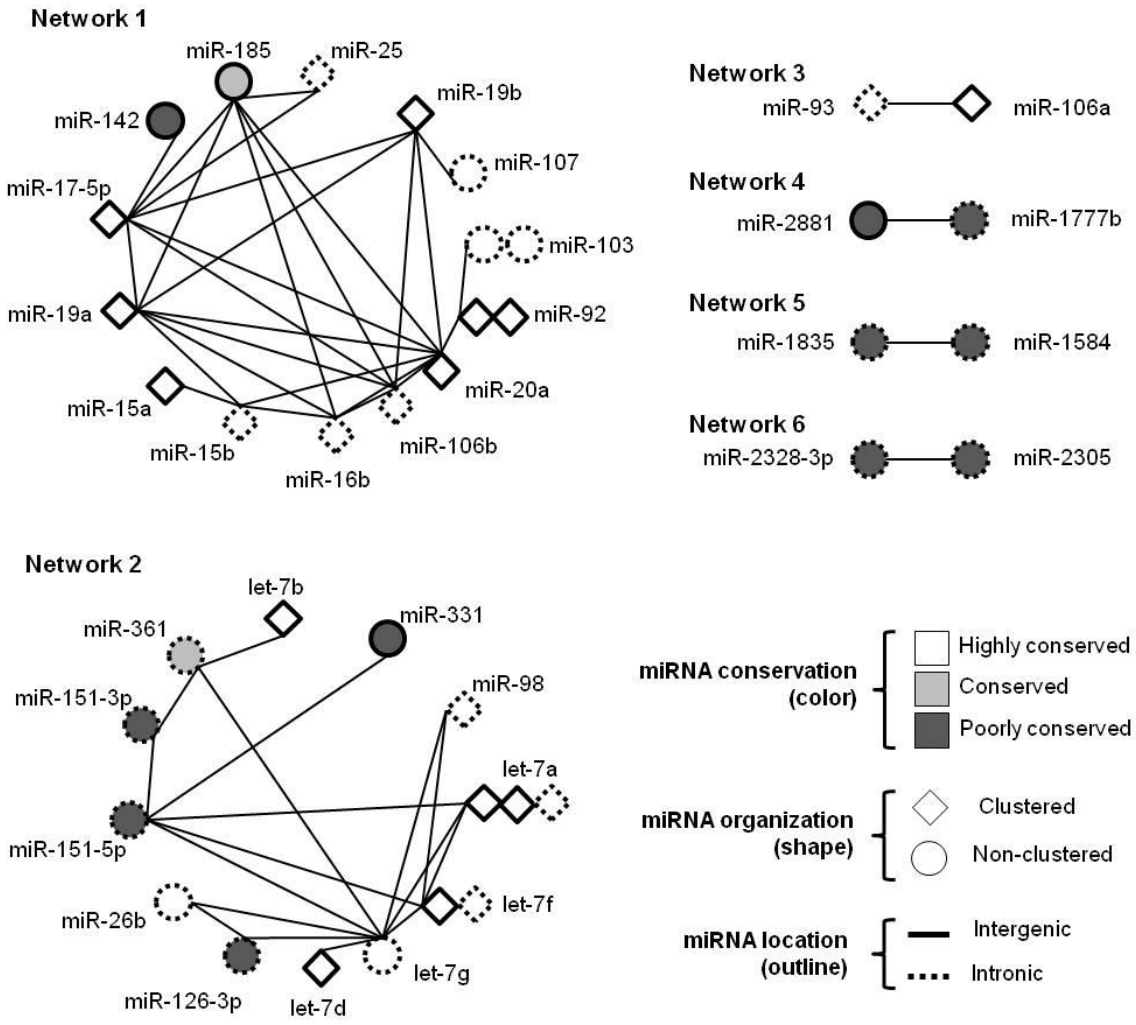

Figure 4.6 Relevant networks of AT core miRNAs.

miRNAs with connecting lines have highly correlated ($R^2 > 0.95$) expression patterns. miRNAs represented by multiple shapes indicates that they are coded at more than one genomic location. AT core miRNAs consist of miRNAs detected in the subcutaneous adipose tissue of all steers.

For a full description of all miRNA clusters expressed in adipose tissue see Table 4.A1. Quantitative RT-PCR confirmed ($p < 0.001$) the correlated expression of miR-19a and miR-19b in Network 1 and supported the microarray

findings of coordinated expression among miRNAs that are members of the same network (Figure 4.7).

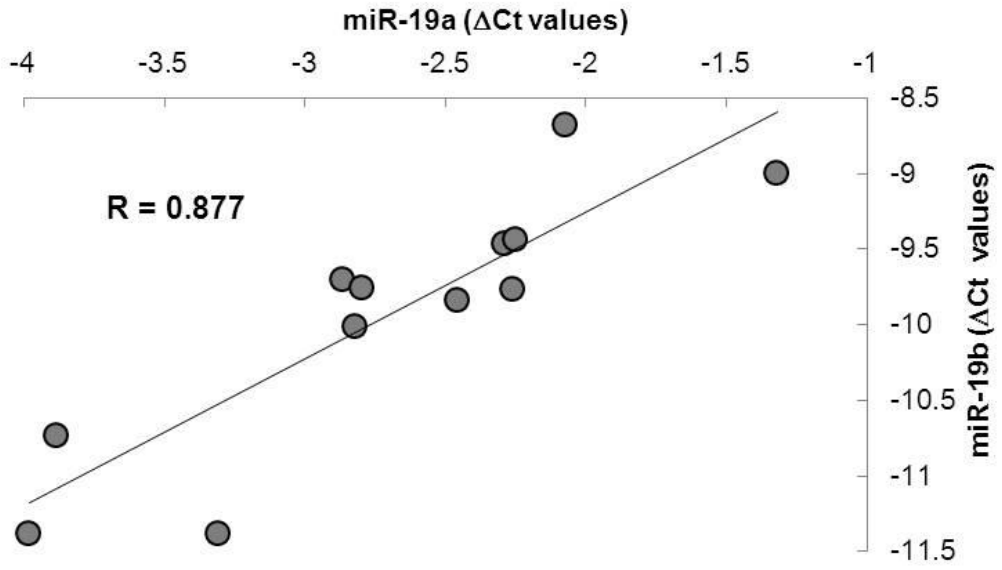

Figure 4.7 Expression levels of miR-19a and miR-19b by qRT-PCR.

Delta Ct values were calculated as follow: ΔCt miR-19a = Ct miR-19a – Ct miR-181a and ΔCt miR-19b = Ct miR-19b – Ct miR-181a.

4.3.5 AT core miRNAs hosted in protein coding genes

A total of 60 AT core miRNAs were located inside 60 protein coding genes. Most of them were intronic (n = 57), two were exonic and one was a mirtron. It is important to note that some of these genes host more than one miRNAs such as minichromosome maintenance complex component 7 (*MCM7*) which contains miR-106b, 93 and 25. Also, some miRNAs are inside introns of more than one gene as is the case for miR-103 within the introns of pantothenate kinase 2 (*PANK2*) and pantothenate kinase 3 (*PANK3*). Therefore the biogenesis

intronic miRNAs does not always follow the rule of a host gene originating only one intronic miRNA or an intronic miRNA being generated by only one host gene. Intronic miRNAs and their host genes may function in common in the same pathways as their expression might be coordinated. Therefore, functional analysis of genes possessing intronic miRNAs may reveal the biological functions of their miRNAs. IPA® software (http://www.ingenuity.com/) mapped 54 out of 60 genes hosting AT core miRNAs to the Ingenuity Knowledge Base revealing that these miRNAs are hosted by genes coding for kinases (n = 6), phosphatases (n = 2), other enzymes (n = 8), ion channel proteins (n = 2), transcription regulators (n = 7), a translation regulator (n = 1), transporters (n = 4), and other proteins (n = 24). These genes were further submitted to a Core analysis (functional analysis) that showed that 41 out of the 54 genes were associated ($p < 0.05$) with 25 categories of IPA molecular and cellular functions (Figure 4.8). The other 13 genes were not associated with IPA functions.

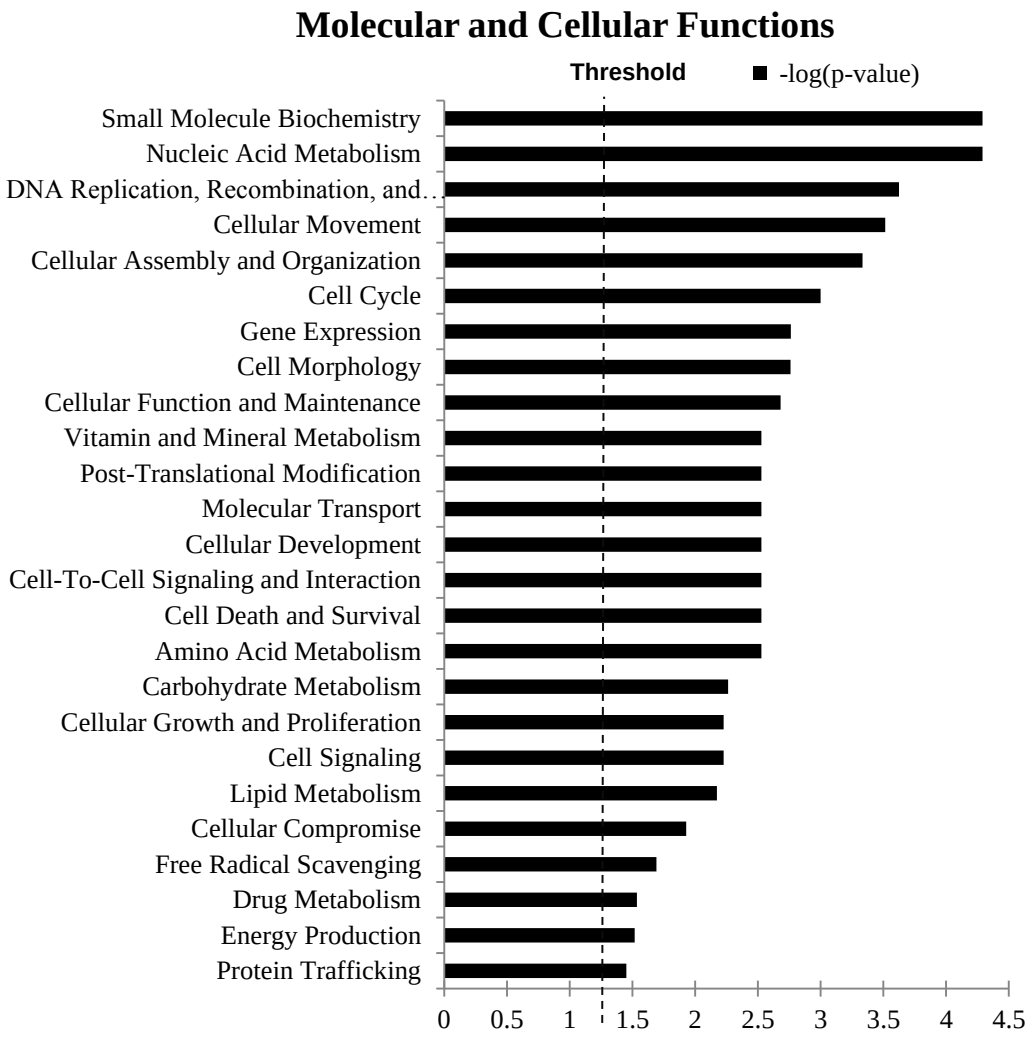

Figure 4.8 Molecular and cellular functions of protein coding genes that host AT core miRNAs.

The likelihood of the association between the genes and a biological function is represented as –log(p-value), with large bars being more significant than short bars. The vertical line indicates the cutoff for significance (p-value of 0.05). AT core miRNAs consist of miRNAs detected in the subcutaneous adipose tissue of all steers

Among the significant functions, several were related to general cellular metabolism such as small molecule biochemistry, cellular movement, cellular assembly and organization, cell cycle among others. A total of 5 genes were associated with lipid metabolism: E1A binding protein p300 (*EP300*), peroxisome proliferator-activated receptor gamma, coactivator 1 beta (*PPARGC1B*), platelet-derived growth factor receptor, beta polypeptide (*PDGFRB*), protein tyrosine kinase 2 (*PTK2*), and sterol regulatory element binding transcription factor 2 (*SREBF2*) (Table 4.1). Each of these genes hosted a single miRNA and a functional analysis indicated that these five genes were involved together in several aspects of lipid metabolism (Table 4.1).

Table 4.1 Functional roles of genes hosting adipose core miRNAs in lipid metabolism

Functions Annotation	p-Value	Molecules
concentration of lipid	5.72E-07	*EP300* (miR-1281), *PDGFRB* (miR-1777b), *PPARGC1B* (miR-378), *PTK2* (miR-151-5p), *SREBF2* (miR-33a)
concentration of phosphatidic acid	4.74E-06	*EP300* (miR-1281), *PDGFRB* (miR-1777b), *PTK2* (miR-151-5p)
quantity of phosphoinositide	1.59E-04	*PDGFRB* (miR-1777b), *PTK2* (miR-151-5p)
quantity of myristic acid	9.03E-04	*PPARGC1B* (miR-378)
quantity of sn-glycero-3-phosphocholine	9.03E-04	*EP300* (miR-1281)
oxidation of lipid	1.47E-03	*PPARGC1B* (miR-378), *SREBF2* (miR-33a)
concentration of palmitic acid	1.81E-03	*PPARGC1B* (miR-378)
concentration of stearic acid	1.81E-03	*PPARGC1B* (miR-378)
concentration of cholesterol	2.98E-03	*PPARGC1B* (miR-378), *SREBF2* (miR-33a)
quantity of phosphatidylinositol-3-phosphate	3.01E-03	*PDGFRB* (miR-1777b)
storage of cholesterol	4.51E-03	*SREBF2* (miR-33a)
uptake of palmitic acid	4.51E-03	*PPARGC1B* (miR-378)
metabolism of membrane lipid derivative	5.82E-03	*PDGFRB* (miR-1777b), *SREBF2* (miR-33a)
quantity of phosphatidylinositol-3,4,5-triphosphate	9.00E-03	*PTK2* (miR-151-5p)
synthesis of phosphatidylinositol-3,4,5-triphosphate	1.05E-02	*PDGFRB* (miR-1777b)
oxidation of palmitic acid	1.20E-02	*PPARGC1B* (miR-378)
accumulation of cholesterol	1.47E-02	*SREBF2* (miR-33a)
synthesis of cholesterol	1.73E-02	*SREBF2* (miR-33a)
synthesis of lipid	2.15E-02	*PDGFRB* (miR-1777b), SREBF2 (miR-33a)
homeostasis of cholesterol	2.36E-02	*SREBF2* (miR-33a)
hydrolysis of phosphatidylinositol	2.74E-02	*PDGFRB* (miR-1777b)

E1A binding protein p300 (*EP300*), peroxisome proliferator-activated receptor gamma, coactivator 1 beta (*PPARGC1B*), platelet-derived growth factor receptor, beta polypeptide (*PDGFRB*), protein tyrosine kinase 2 (*PTK2*), and sterol regulatory element binding transcription factor 2 (*SREBF2*).

The relationship between genes involved in lipid metabolism and their intronic miRNAs was further analyzed by comparing their expression using qRT-PCR. The analysis included *EP300* (miR-1281), *PPARGC1B* (miR-378), *PTK2* (miR-151-5p) and *SREBF2* (miR-33a). The expression of the pair *PDGFRB* (miR-1777b) was not assessed as it was not possible to design primers for miR-1777b owing to its very high GC content. A Pearson correlation analysis showed that expression of miR-1281 and miR-33a was correlated ($p<0.05$) with their host gene, while no correlation ($p>0.05$) with the host gene was observed for miR-378 and 151-5p (Table 4.2).

Table 4.2 Correlation analysis of host genes and intronic miRNAs expression by qRT-PCR

Gene	miRNA	R	p-value
EP300	miR-1281	0.624	0.030
PPARGC1B	miR-378	0.193	0.554
SREBF2	miR-33a	0.635	0.027
PTK2	miR-151-5p	0.001	0.996

E1A binding protein p300 (EP300), peroxisome proliferator-activated receptor gamma, coactivator 1 beta (PPARGC1B), sterol regulatory element binding transcription factor 2 (SREBF2) and protein tyrosine kinase 2 (PTK2). Correlation was significant at $p < 0.05$

4.3.6 Bovine specific miRNAs

Among the 57 poorly conserved AT core miRNAs, a total of 23 had seed regions (7 nucleotides at the 5' end of miRNA) that are found only in bovine (Table 4.3). Three bovine specific miRNAs (miR-1584, miR-2412 and miR-2374) had different sequences; however, they shared the same seed region "UGGGGCU". Except for miR-425-5p, all bovine specific miRNAs did not cluster and 13 out of 23 were located in intergenic regions. Expression of bovine specific miRNAs varied considerably and was as much as 70 fold higher for miR-2478 than that of miR-2882. Similarly, the numbers of predicted targets for each bovine specific miRNA were highly variable (Table 4.3).

Table 4.3 Bovine specific miRNAs expressed in adipose tissue of all steers

microRNAs	Chr.	Cluster	Location	Host gene[1]	miRNA expression	Predicted Targets[2]	Seed region[3]
bta-miR-2478	chr9	no	intergenic	-	10.90 ± 0.63	116	UAUCCCA
bta-miR-126-3p	chr11	no	intronic	*EGFL7*	7.71 ± 0.63	26	GUACCGU
bta-miR-2305	chr13	no	intronic	*RIN2*	6.09 ± 1.19	109	GGGGGUG
bta-miR-2328-3p	chr18	no	intronic	*ZNF821*	5.39 ± 1.22	144	CCCCCUC
bta-miR-1584	chr3	no	intronic	*TAGLN2*	5.13 ± 0.85	135	UGGGGCU
bta-miR-2888	chr21	no	intergenic	-	4.60 ± 0.98	93	GUGGGGU
bta-miR-199c	chr19	no	intronic	*NUP88*	4.01 ± 0.38	704	ACAGUAG
bta-miR-2881	chr7	no	intergenic	-	3.95 ± 0.88	59	GGGCGGG
bta-miR-2332	chr19	no	intronic	*UTP6*	3.39 ± 0.39	392	GGUUUAA
bta-miR-2412	chr3	no	intergenic	-	3.34 ± 0.99	135	UGGGGCU
bta-miR-2455	chr7	no	intergenic	-	3.07 ± 1.14	413	CUGUGCU
bta-miR-2316	chr15	no	intergenic	-	2.53 ± 0.76	8	CUCCGGC
bta-miR-2374	chr22	no	intergenic	-	1.98 ± 0.83	135	UGGGGCU
bta-miR-2483	chrX	no	intergenic	-	1.62 ± 1.12	381	AACAUCU
bta-miR-2474	chr8	no	intronic	*SHB*	1.61 ± 0.96	5	ACCGGGC
bta-miR-425-5p	chr22	yes	intergenic	-	1.61 ± 0.66	74	UGACACG
bta-miR-2892	chr12	no	intergenic	-	1.52 ± 0.65	3	GCGACGG
bta-miR-1434	chr7	no	intronic	*EEF2*	1.02 ± 0.57	1134	AAGAAAU
bta-miR-2391	chr26	no	intergenic	-	0.55 ± 0.40	2732	AAAAAAA
bta-miR-2898	chr8	no	intergenic	-	0.37 ± 0.50	134	GGUGGAG
bta-miR-2424	chr5	no	intronic	*NCAPD2*	0.29 ± 0.49	225	GAUCUUU
bta-miR-2885	chr29	no	intergenic	-	0.20 ± 0.34	27	GGCGGCA
bta-miR-2882	chr7	no	exonic	*SMARCA4*	0.15 ± 0.23	19	GCCCGGG

[1]EGF-like-domain, multiple 7 (*EGFL7*), Ras and Rab interactor 2 (*RIN2*), zinc finger protein 821 (*ZNF821*), transgelin 2 (*TAGLN2*), nucleoporin 88kDa (*NUP88*), UTP6, small subunit (*SSU*) processome component, homolog (yeast) (*UTP6*), Src homology 2 domain containing adaptor protein B (*SHB*), eukaryotic translation elongation factor 2 (*EEF2*), uncharacterized LOC509171 (*NCAPD2*), SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (*SMARCA4*)

Chr. stands for chromosome location

[2]The amount of targets by miRNA is based on TargetScan prediction tool release 6.2

[3]Includes 7 nucleotides at positions 2-8 starting at the 5'end of the mature miRNA sequence

The potential regulatory functions of the 23 bovine specific miRNAs expressed in all cattle were investigated by performing individual target prediction analyses (TargetScan). Only targets predicted with total context^{+} scores $\leq$ -0.3 were selected to reduce false positive predictions (Xiao et al., 2012). As miR-1584, miR-2412 and miR-2374 have the same seed region, they were analyzed as one entry "miR-1584/2412/2374". An IPA® Core Analysis was performed on predicted targets of each bovine specific miRNA in order to identify their function and elucidate the potential regulatory roles of these miRNAs. In combination, the 23 core adipose bovine specific miRNAs were predicted to regulate genes associated ($p<0.05$) with 25 different molecular and cellular functions (Figure 4.A2). Fifteen of these were potential regulators of pathways related to lipid metabolism, carbohydrate metabolism and/or energy production as one or more of their predicted targets were involved ($p<0.05$) with these functions (Figure 4.9). Additionally, these 15 miRNAs were also associated with predicted targets involved in other functions including cell-to-cell signalling and interaction. miR-2892 was the only bovine specific miRNA that was not associated with any predicted molecular and cellular function.

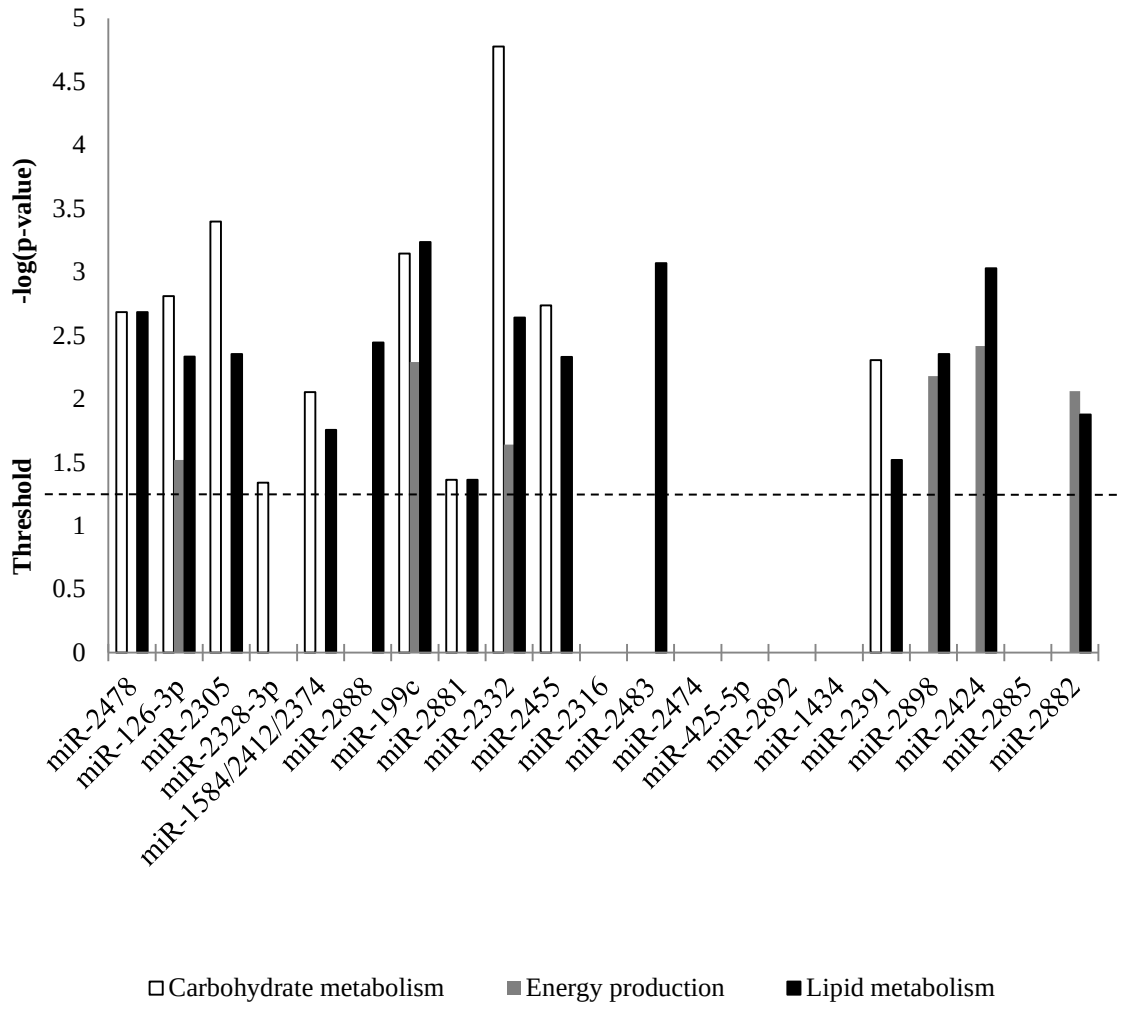

Figure 4.9 Association of predicted targets from bovine specific miRNAs to energy production, carbohydrate, or lipid metabolism.

The likelihood of the association between the genes and a biological function is represented as –log(p-value), with large bars being more significant than short bars. The horizontal line indicates the cutoff for significance (p-value of 0.05). AT core miRNAs consist of miRNAs detected in the subcutaneous adipose tissue of all steers.

4.4 DISCUSSION

Knowledge of miRNA biology has been growing consistently over the past few years, but a clear understanding of their role in the regulation of cellular metabolism remains elusive. miRNAs are a group of diverse regulatory molecules with potential to target hundreds of genes (Thomas et al., 2010) and integrate complex molecular regulatory networks. Studies have focused on identifying differential expression of miRNAs in adipose of individuals submitted to opposite conditions such as low fat vs. high fat diet treatments (Chartoumpekis et al., 2012) or lean vs. obese individuals (Hurtado del Pozo et al., 2011) which reveals only particular aspects of miRNA biology in adipose tissue. However, in this study we aimed to perform a more holistic approach by focusing on the genomic context features of miRNA genes and their global expression under physiological adipose tissue conditions.

To date, the amount of miRNAs identified in bovine (n=755) is the third highest in mammals following those of human (n=2042) and mice (n=1281) (miRBASE, 2012). Bovine miRNAs are not evenly distributed within the genome as numbers may vary up to 10 fold among individual chromosomes (Figure 4.1). Chromosome X of several mammalian species has been reported to have a higher miRNA density (# miRNA/chromosome size) as compared to those associated with autosomes (Guo et al., 2009). Interestingly, chromosome X had the highest number of AT core miRNAs expressed in our study, suggesting a relevant role of this chromosome and its miRNAs in the function of adipose tissue. This variation in distribution may contribute to the differences in fat metabolism observed

between male and female mammals in a manner analogous to the proposed role that miRNAs on chromosome X have on immune functions between female vs. male humans and mice (Pinheiro et al., 2011). So far, no bovine miRNA gene has been reported on Chromosome Y according to miRBase. This could reflect the fact that the reference bovine genome (UMD3.1) was generated from a cow. However, it is expected that few miRNAs are coded in the bovine chromosome Y as other mammals such as mice have none (0/1281 miRNAs) and humans only have 2 out of 2042 miRNAs coded on chromosome Y (miRBASE, 2012).

A total of 155 miRNAs were consistently detected in samples of subcutaneous adipose tissue from all individuals. However, it is probable that these miRNAs are not exclusively associated with adipocytes as adipose tissue is also composed of preadipocytes, macrophages, endothelial cells and stem cells. Therefore the results from this study reflect the possible role of miRNA in the regulation of adipose tissue as a whole.

The core adipose miRNAs were heterogeneous in terms of genomic context and conservation status. A large portion (44.5%) of the miRNAs that integrated the core adipose miRNAs was organized in clusters (Figure 4.2). They differed from non-clustered miRNAs due to their close proximity on the genome (< 10kb), indicating that they may be subject to coordinated expression, by sharing the same promoter (Yu et al., 2006). Interestingly, adipose core miRNAs that organized in clusters were both from the same (e.g. cluster including let-7a, -7d, -7f in chr. 8) or different miRNA families (e.g. cluster including miR-17-3p, -18a, -19a, -20a, -19b, -92 in chr. 12). The presence of miRNAs from different

families in the same cluster suggests that coordinated expression of these miRNAs enables regulation of different target messenger RNAs, and consequently to control expression of different genes within a common pathway (Yuan et al., 2009; Olena and Patton, 2010). Adipose core miRNAs were coded mainly in intergenic regions (59.5%); however, intronic miRNAs were also numerous (38.7%).

miRNAs encoded in intronic regions are generally co-expressed with the host gene and may represent an auto-regulatory mechanism (Bosia et al., 2012). They may also support host gene expression by either down-regulating antagonist genes or fine-tuning the expression of genes (miRNA targets) that co-operate with the host gene in specific biological processes (Lutter et al., 2010). Exploring the functions of genes hosting intronic miRNAs may provide insight into the role of these molecules in bovine adipose tissue.

The behaviour of core adipose miRNAs followed a trend in which expression was inversely correlated to miRNA variation among different samples. miRNAs highly expressed had more consistent levels among samples, while miRNAs lowly expressed were more variable (Figure 4.3) which resembles the behaviour of genes as observed in a study that profiled the expression of 11,335 transcripts in lymphoblastoid cell lines from 210 humans (Fan et al., 2009). The nature of the function performed by each gene is likely to influence the level of expression variation in the population (Shalek et al., 2013), which might also occur to miRNAs.

In addition, highly conserved miRNAs expressed higher than poorly conserved miRNAs (Figure 4.4). This result supports the idea that consistently high expressed miRNAs play critical roles in the regulation of cellular processes in bovine adipose tissue as they tend to be maintained by natural selection in most vertebrates (highly conserved miRNAs). This idea is also corroborated by the fact that highly expressed miRNAs are under intense selective pressure to maintain sequence uniformity while lowly expressed miRNAs tend to have much lower selection pressure being more prone to evolve rapidly (Liang and Li, 2009).

Conservation status of bovine miRNAs was also associated with the number of predicted targets (Figure 4.5) as highly conserved miRNAs had approximately 3 times more targets than poorly conserved miRNAs. Our results are supported by a human study that reported that highly conserved miRNAs had higher repression efficiency as compared to less conserved miRNAs (Huang and Gu, 2011). The behavior of highly conserved miRNAs was similar to that of clustered miRNAs in terms of expression (Figure 4.4) and amount of predicted targets (Figure 4.5). This is explained by the fact that highly conserved miRNAs tended to be organized in clusters, while less conserved miRNAs were more unclustered. The association between miRNA conservation and miRNA genomic organization was evident when miRNA relevant networks analysis was performed (Figure 4.6).

It is challenging to determine the scope of miRNA regulation in a biological process as each miRNA may target several mRNAs and each mRNA may be regulated by multiple miRNAs (Lai et al., 2012). Evidence shows that

miRNAs may work in a coordinated fashion to regulate gene expression in complex networks (Siengdee et al., 2013). However, identifying miRNA functional networks is difficult as computational prediction tools are not accurate enough to identify only the true miRNA targets. Therefore, relying on miRNA expression data is an important piece of information to determine which miRNAs are functioning in a coordinated manner to regulate a certain biological process.

Our study identified six relevant miRNA networks using miRNA expression data, suggesting that miRNAs regulate gene expression in bovine adipose tissue in a coordinated fashion. Although it is not possible to define the exact roles of each miRNA network identified in adipose tissue, some of the miRNAs identified have already been studied. For example miR-17-5p, 19a, 20a, 19b and 92 (Network 1) are part of the cluster 17~92 which was shown to be up-regulated during the clonal expansion step of adipocyte differentiation of 3T3-l1 cells (Wang et al., 2008). Over-expression of cluster 17-92 accelerated adipocyte differentiation and triglyceride accumulation by targeting and negatively regulating the gene coding tumor suppressor retinoblastoma-like 2 (p130) (Wang et al., 2008). miR-103 and miR-107 were found to be up-regulated in adipocyte differentiation (Xie et al., 2009a) and have been reported to play a role in the regulation of insulin sensitivity (Trajkovski et al., 2011). In this context it is likely that network 1 is involved in the regulation of the development and metabolism within bovine adipose tissue. Similarly, network 2 has miRNAs known to regulate adipogenesis, such as the highly expressed let-7 family which has been reported to

regulate the transition from clonal expansion to terminal differentiation of adipocytes (Sun et al., 2009).

The smaller networks (4, 5, and 6) may play a role in the regulation of bovine adipose tissue in more limited ways as they were poorly conserved and some were bovine specific (miR-2881, -2328, and 2305). Therefore, these miRNA regulatory networks may contribute to species-specific metabolic characteristics in adipose tissue of bovines or other ruminants as previous studies have pinpointed that bovine adipogenesis is different than that of other species including human and mice (Taniguchi et al., 2008; Dodson et al., 2010a). Functional studies and experimental validation of target genes of bovine specific miRNAs may shed some light on their specific roles in the function of bovine adipose tissue. Many other AT core miRNAs (n = 33) that were not included in the relevant networks were also organized in clusters suggesting that their expression might also be coordinated with other members of the cluster.

Coordinated expression does not only occur with miRNAs, but intronic miRNAs expression may also be coordinated with host genes (Baskerville and Bartel, 2005). This study revealed that 39% of core adipose miRNAs were coded within introns of host genes (Figure 4.2) involved in a multitude of cellular and molecular functions (Figure 4.8). Defining the function of these host genes may help identify the regulatory role of intronic miRNAs, as they may assist the expression/function of the host gene (Barik, 2008) or be involved in the same biological process.

In the context of adipose tissue, five intronic miRNAs were identified as being associated with genes potentially involved in lipid metabolism (Table 4.1). In fact, some of these miRNAs have already been reported to play a role in adipogenesis or lipid metabolism including miR-378 which was found up-regulated in steers with high levels of subcutaneous fat (Jin et al., 2010). This miRNA has also been reported to increase the size of lipid droplets in mice ST2 cells when over-expressed (Gerin et al., 2010). Similarly, miR-33a was found to be involved in the regulation of β-oxidation of fatty acids, cholesterol homeostasis and insulin signalling (Dávalos et al., 2011). miR-33a expression in subcutaneous adipose tissue was significantly correlated with its host gene *SREBF2* (Table 4.2), a transcription factor that controls the expression of other genes involved with cholesterol metabolism (Horton, 2002). These findings are in agreement with findings that show coordinated expression of miR-33a and *SREBF2* over a range of tissue types (Najafi-Shoushtari et al., 2010). miR-1281 also showed a coordinated expression pattern with its host gene (*EP300*). It is possible that miR-1281 might play a role in adipogenesis as EP300 is a transcriptional co-activator of CCAAT/enhancer binding protein α (C/EBPα) (Erickson et al., 2001), which is a critical transcription factor for adipocyte differentiation (Tang and Lane, 2012).

Expression of miR-378 and miR151-5p did not correlate with expression of their host genes, but this observation does not rule out the possibility that coordinated expression could still occur. For instance, miR-378 in bovine is coded in two different genomic locations, one is located in an intron of *PPARGC1B* gene in chromosome 7 (precursor miR-378-1) and the other in an intergenic

region in chromosome 4 (precursor miR-378-2). Therefore, *PPARGC1B* expression is not the only source for miR-378, which may explain why a significant correlation was not found between miR-378 and *PPARGC1B* levels. PTK2 is a signaling molecule that can promote cell motility (Mitra et al., 2005) and its expression has been reported to be coordinated with miR-151 in hepatocellular carcinoma tissue samples (Ding et al., 2010). This could be why in adipose tissue of uniform and healthy steers the coordinated expression between miR-151-5p and *PTK2* was not observed. Others have shown that coordinated expression between intronic miRNAs and host gene does not always occur as intronic miRNAs can also be independently transcribed from host genes (Isik et al., 2010; He et al., 2012a).

The group of poorly conserved miRNAs identified in this study includes a subset termed as bovine specific miRNAs, which represent miRNAs with a seed sequence unique to *Bos taurus*. They accounted for approximately 15% of the bovine core adipose miRNA and were typically non-clustered (Table 4.3). miRNAs are rarely lost when an animal lineage acquires them (Wheeler et al., 2009), therefore bovine specific miRNAs are likely to be evolutionarily younger than highly conserved miRNAs as they are not found in other mammalian groups such as primates or rodents. The species specificity of these miRNAs suggests that they may perform a regulatory function that is unique to adipose tissue metabolism in *Bos taurus* or other ruminants. In general, species specific miRNAs tend to be less expressed than highly conserved miRNAs, but bovine specific miR-2478 was the most highly expressed miRNA in all animals from our study

suggesting it has an established role in bovine adipose tissue. In addition, miRNAs highly expressed are reported to be under a high selective pressure to maintain their sequence unaltered in comparison to lowly expressed miRNAs that are rapidly evolving with frequent mutations (Liang and Li, 2009). Analysis of predicted targets for bovine specific miRNA revealed that 17 out of 23 of these core adipose miRNAs may be involved in different aspects of adipose tissue energy balance by targeting genes involved in lipid metabolism, carbohydrate metabolism and/or energy production (Figure 4.9).

4.5 CONCLUSIONS

In conclusion, the core adipose miRNAs are widely spread on bovine chromosomes, and genomic context features such as miRNA organization in clusters and miRNA evolutionary conservation were associated with their expression and quantity of predicted targets. Core adipose miRNAs are likely to work collectively as their expression revealed that 34 of them are part of six regulatory networks, with each displaying a unique coordinated behavior. Another instance of coordinated expression was identified between two intronic miRNAs and their host genes involved in lipid metabolism (miR-33a/*SREBF2* and miR-1281/*EP300*), suggesting these miRNAs regulate the lipid metabolism pathway that involves their host genes. The specific functions of miRNAs are largely unknown, especially for species specific miRNAs. The bovine specific miRNAs expressed in all animals accounted for almost 15% of the core adipose miRNAs. They are likely to be involved in aspects of adipose tissue metabolism that are

unique to bovines and or ruminants with the predicted targets suggesting that 17 of them play a role in regulating the energetic balance of bovine adipose tissue. The results obtained in this study expand our understanding on miRNA functions and behaviour in bovine adipose tissue which might help the development in the future of new strategies to manipulate adiposity in beef cattle improving meat quality and animals productivity.

4.6 APPENDIX

Figure 4.A1. miRNA gene density in each bovine chromosome

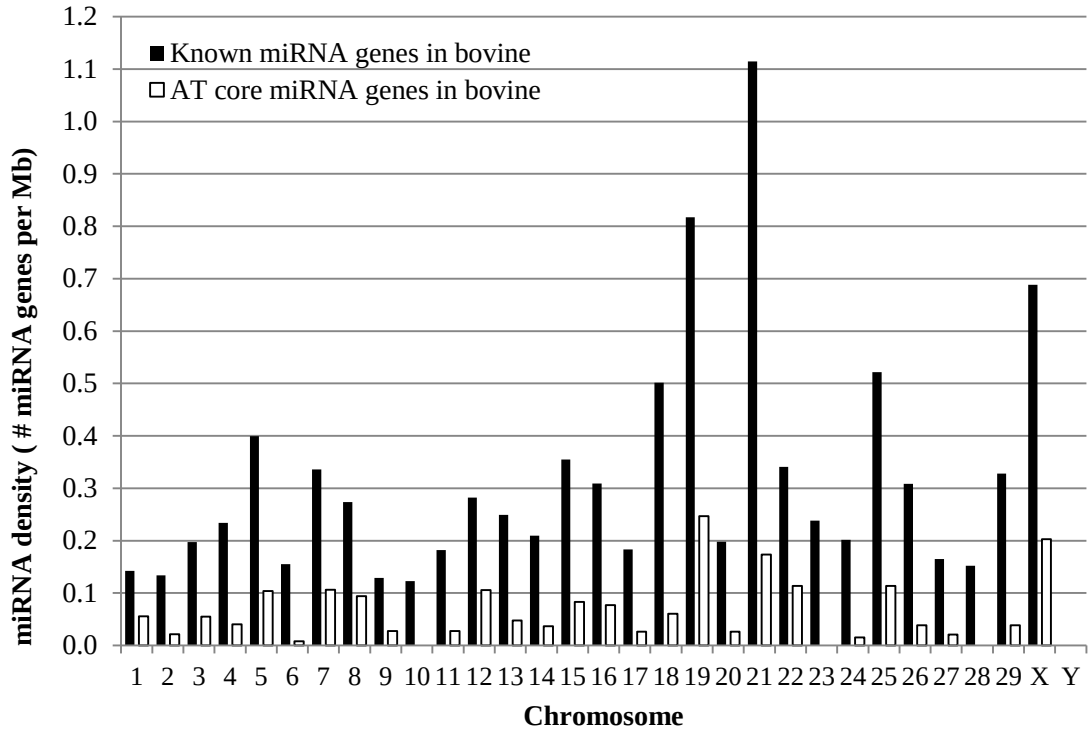

Figure 4.A2. Molecular and cellular functions of predicted targets of bovine specific miRNAs

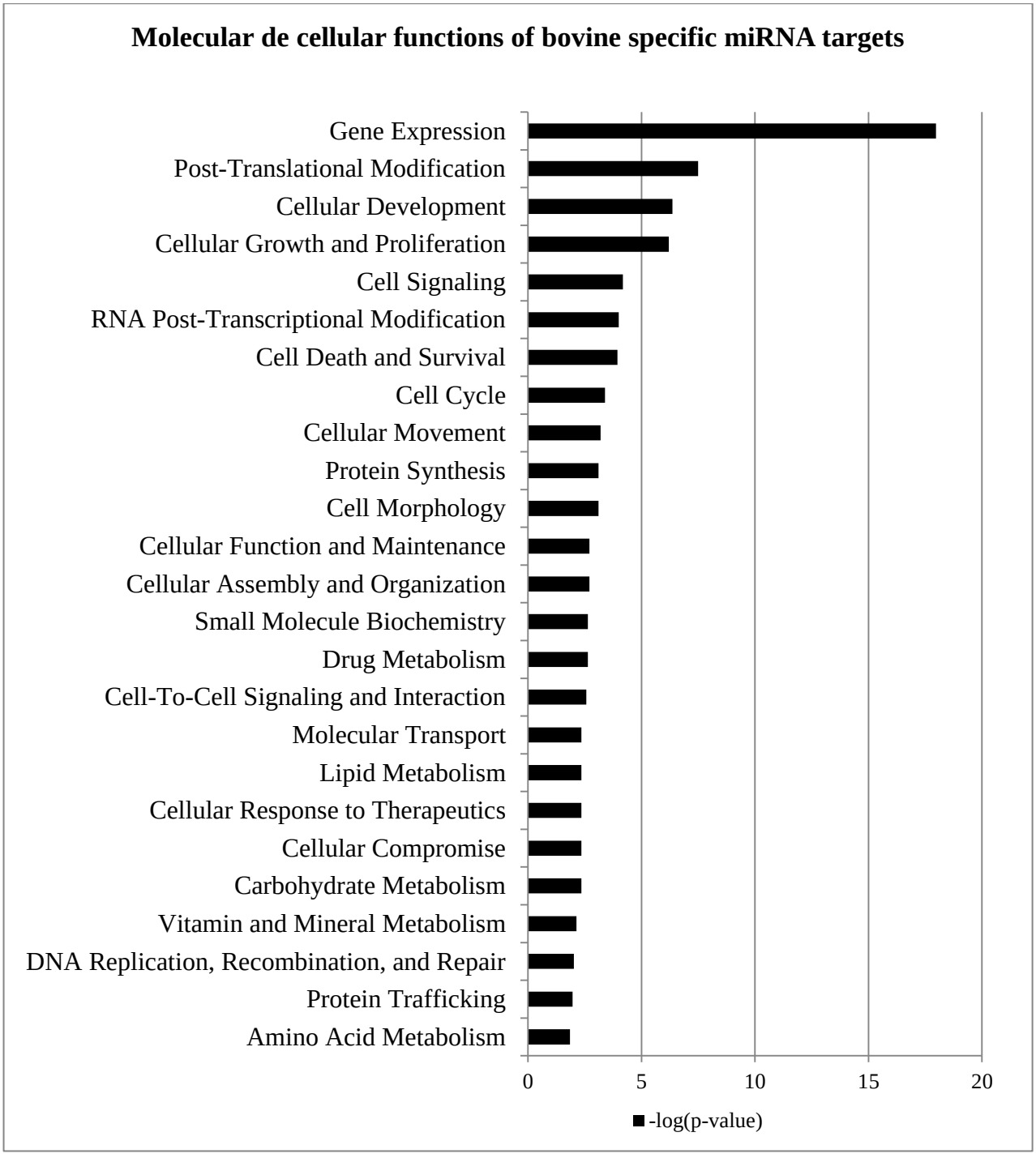

Table 4.A1. Genomic context, conservation and expression of AT core miRNAs

#	miRNAs	miR precursor	Chr.	S.	Start	End	Conservation	Cluster	Location	Host Gene
1	bta-let-7a	bta-let-7a-1	8	+	86884872	86884951	Highly conserved	yes	intergenic	
2		bta-let-7a-2	15	-	33347569	33347640	Highly conserved	yes	Intronic	LOC100848689
3		bta-let-7a-3	5	+	117119385	117119458	Highly conserved	yes	intergenic	
4	bta-let-7b	bta-let-7b	5	+	117120185	117120265	Highly conserved	yes	intergenic	
5	bta-let-7c	bta-let-7c	1	-	19930459	19930542	Highly conserved	yes	intergenic	
6	bta-let-7d	bta-let-7d	8	+	86887431	86887517	Highly conserved	yes	intergenic	
7	bta-let-7e	bta-let-7e	18	+	58015036	58015114	Highly conserved	yes	intergenic	
8	bta-let-7f	bta-let-7f-1	8	+	86885225	86885311	Highly conserved	yes	intergenic	
9		bta-let-7f-2	X	-	96383532	96383614	Highly conserved	yes	intronic	HUWE1
10	bta-let-7g	bta-let-7g	22	+	49189340	49189422	Highly conserved	no	intronic	WDR82
11	bta-let-7i	bta-let-7i	5	-	51209081	51209164	Highly conserved	no	intergenic	
12	bta-miR-100	bta-mir-100	15	-	33353395	33353470	Highly conserved	yes	intronic	LOC100848689
13	bta-miR-101	bta-mir-101-1	3	+	80666417	80666499	Highly conserved	no	exonic	LOC100139603
14		bta-mir-101-2	8	-	39940832	39940910	Highly conserved	no	intronic	RCL1
15	bta-miR-103	bta-mir-103-1	20	-	189857	189928	Highly conserved	no	intronic	PANK3
16		bta-mir-103-2	13	-	51742422	51742497	Highly conserved	no	intronic	PANK2
17	bta-miR-106a	bta-mir-106a	X	-	17918527	17918607	Highly conserved	yes	intergenic	
18	bta-miR-106b	bta-mir-106b	25	+	36892046	36892125	Highly conserved	yes	intronic	MCM7
19	bta-miR-107	bta-mir-107	26	-	11287994	11288074	Highly conserved	no	Intronic	PANK1
20	bta-miR-10a	bta-mir-10a	19	+	38549254	38549362	Highly conserved	yes	intergenic	
21	bta-miR-10b	bta-mir-10b	2	-	20797606	20797704	Highly conserved	no	intergenic	
22	bta-miR-1	bta-mir-1-1	13	-	55237544	55237619	Highly conserved	no	intergenic	
23		bta-mir-1-2	24	+	34841096	34841180	Highly conserved	yes	intergenic	
24	bta-miR-1224	bta-mir-1224	1	-	83546862	83546947	Pooly conserved	no	mirtron	VWA5B2
25	bta-miR-125b	bta-mir-125b-1	15	-	33298815	33298902	Highly conserved	no	intronic	LOC100848689
26		bta-mir-125b-2	1	-	19881347	19881431	Highly conserved	no	intergenic	
27	bta-miR-126-3p	bta-mir-126	11	+	104131409	104131481	Pooly conserved	no	Intronic	EGFL7
28	bta-miR-126-5p						Pooly conserved	no	Intronic	
29	bta-miR-127	bta-mir-127	21	+	67429744	67429838	Conserved	yes	intergenic	
30	bta-miR-128	bta-mir-128-1	2	-	62007752	62007833	Highly conserved	no	intronic	R3HDM1
31		bta-mir-128-2	22	+	9738598	9738681	Highly conserved	no	intergenic	ARPP21
32	bta-miR-1281	bta-mir-1281	5	+	112802157	112802210	Pooly conserved	no	intronic	EP300
33	bta-miR-130a	bta-mir-130a	15	+	82197711	82197799	Highly conserved	no	intergenic	
34	bta-miR-1343	bta-mir-1343	15	+	66264848	66264925	Pooly conserved	no	Intronic	PDHX
35	bta-miR-136	bta-mir-136	21	+	67431418	67431509	Conserved	yes	intergenic	
36	bta-miR-139	bta-mir-139	15	-	52956182	52956249	Highly conserved	no	Intronic	PDE2A
37	bta-miR-140	bta-mir-140	18	+	37088137	37088230	Pooly conserved	no	intronic	WWP2
38	bta-miR-142-5p	bta-mir-142	19	-	9527305	9527391	Pooly conserved	no	intergenic	
39	bta-miR-143	bta-mir-143	7	+	62809304	62809404	Highly conserved	yes	intergenic	
40	bta-miR-1434	bta-mir-1434	7	+	21255389	21255461	Pooly conserved	no	intronic	EEF2
41	bta-miR-144	bta-mir-144	19	-	20797022	20797105	Highly conserved	yes	intergenic	
42	bta-miR-145	bta-mir-145	7	+	62810733	62810820	Highly conserved	yes	intronic	LOC100849059
43	bta-miR-148a	bta-mir-148a	4	+	70414251	70414318	Highly conserved	no	intergenic	
44	bta-miR-148b	bta-mir-148b	5	-	25849764	25849853	Highly conserved	no	intronic	COPZ1
45	bta-miR-151-3p	bta-mir-151	14	+	4030319	4030387	Pooly conserved	no	Intronic	PTK2
46	bta-miR-151-5p						Pooly conserved	no	Intronic	
47	bta-miR-155	bta-mir-155	1	-	10227277	10227339	Highly conserved	no	intergenic	
48	bta-miR-1584	bta-mir-1584	3	+	9881758	9881830	Pooly conserved	no	intronic	TAGLN2

49	bta-miR-15a	bta-mir-15a	12	-	19596346	19596428	Highly conserved	yes	intergenic	
50	bta-miR-15b	bta-mir-15b	1	-	107923379	107923476	Highly conserved	yes	Intronic	SMC4
51	bta-miR-16a	bta-mir-16a	12	-	19596200	19596288	Highly conserved	yes	intergenic	
52	bta-miR-16b	bta-mir-16b	1	-	107923236	107923328	Highly conserved	yes	Intronic	SMC4
53	bta-miR-17-3p	bta-mir-17	12	+	66226554	66226637	Pooly conserved	yes	intergenic	
54	bta-miR-17-5p						Highly conserved	yes	intergenic	
55	bta-miR-1777a	bta-mir-1777a	6	-	107199000	107199075	Pooly conserved	no	Intronic	NSG1
56	bta-miR-1777b	bta-mir-1777b	7	-	63524522	63524601	Pooly conserved	no	intronic	PDGFRB
57	bta-miR-181a	bta-mir-181a-1	16	-	79685932	79686041	Highly conserved	yes	intergenic	
58		bta-mir-181a-2	11	+	95709411	95709520	Highly conserved	yes	intronic	LOC100848407
59	bta-miR-1835	bta-mir-1835	5	-	76049715	76049777	Pooly conserved	no	intronic	RAC2
60	bta-miR-185	bta-mir-185	17	+	74967066	74967144	Conserved	no	intergenic	
61	bta-miR-186	bta-mir-186	3	+	74462295	74462380	Conserved	no	intronic	ZRANB2
62	bta-miR-18a	bta-mir-18a	12	+	66226697	66226767	Highly conserved	yes	intergenic	
63	bta-miR-193a-3p	bta-mir-193a	19	-	18824221	18824301	Highly conserved	no	intergenic	
64	bta-miR-193a-5p						Pooly conserved	no	intergenic	
65	bta-miR-195	bta-mir-195	19	-	27441343	27441429	Highly conserved	yes	intergenic	
66	bta-miR-196a	bta-mir-196a-1	19	+	38496984	38497085	Highly conserved	no	intergenic	
67		bta-mir-196a-2	5	-	26199801	26199885	Highly conserved	no	intergenic	
68	bta-miR-197	bta-mir-197	3	-	33964964	33965044	Conserved	no	intergenic	
69	bta-miR-199a-3p	bta-mir-199a-1	16	-	40491602	40491705	Pooly conserved	yes	intergenic	
70	bta-miR-199a-5p	bta-mir-199a-2	7	-	16508926	16508996	Highly conserved	no	intergenic	
71	bta-miR-199b	bta-mir-199b	11	-	98867657	98867756	Highly conserved	yes	intergenic	
72	bta-miR-199c	bta-mir-199c	19	+	26741713	26741788	Pooly conserved	no	intronic	NUP88
73	bta-miR-19a	bta-mir-19a	12	+	66226836	66226917	Highly conserved	yes	intergenic	
74	bta-miR-19b	bta-mir-19b	12	+	66227133	66227219	Highly conserved	yes	intergenic	
75	bta-miR-204	bta-mir-204	8	-	47259539	47259634	Highly conserved	no	intronic	TRPM3
76	bta-miR-20a	bta-mir-20a	12	+	66227005	66227075	Highly conserved	yes	intergenic	
77	bta-miR-21	bta-mir-21	19	+	11033072	11033143	Highly conserved	no	intergenic	
78	bta-miR-210	bta-mir-210	29	+	51025433	51025530	Pooly conserved	no	intergenic	
79	bta-miR-214	bta-mir-214	16	-	40485807	40485916	Highly conserved	yes	intergenic	
80	bta-miR-22-3p	bta-mir-22	19	-	23382435	23382519	Highly conserved	no	intergenic	
81	bta-miR-22-5p						Pooly conserved	no	intergenic	
82	bta-miR-221	bta-mir-221	X	+	103538835	103538944	Highly conserved	yes	intergenic	
83	bta-miR-223	bta-mir-223	X	-	99936305	99936412	Highly conserved	no	intergenic	
84	bta-miR-224	bta-mir-224	X	-	34664590	34664670	Conserved	yes	intronic	GABRE
85	bta-miR-2288	bta-mir-2288	1	-	139318007	139318083	Pooly conserved	no	intergenic	
86	bta-miR-2304	bta-mir-2304	13	-	28799305	28799378	Pooly conserved	no	Intronic	FRMD4A
87	bta-miR-2305	bta-mir-2305	13	+	39836873	39836938	Pooly conserved	no	Intronic	RIN2
88	bta-miR-2316	bta-mir-2316	15	+	47272310	47272384	Pooly conserved	no	intergenic	
89	bta-miR-2328-3p	bta-mir-2328	18	+	39351757	39351834	Pooly conserved	no	Intronic	ZNF821
90	bta-miR-2332	bta-mir-2332	19	+	18581961	18582029	Pooly conserved	no	Intronic	UTP6
91	bta-miR-2374	bta-mir-2374	22	+	59818212	59818288	Pooly conserved	no	intergenic	
92	bta-miR-2391	bta-mir-2391	26	+	10964297	10964359	Pooly conserved	no	intergenic	
93	bta-miR-23a	bta-mir-23a	7	-	12981970	12982042	Highly conserved	yes	intergenic	
94	bta-miR-23b-3p	bta-mir-23b	8	+	83009615	83009674	Highly conserved	yes	intergenic	
95	bta-miR-2411-3p	bta-mir-2411	3	-	16327516	16327592	Pooly conserved	no	intronic	UBAP2L
96	bta-miR-2412	bta-mir-2412	3	-	18845837	18845914	Pooly conserved	no	intergenic	
97	bta-mir-2424	bta-mir-2424	5	-	104262188	104262267	Pooly conserved	no	intronic	NCAPD2
98	bta-mir-2425-3p	bta-mir-2425	5	+	30251637	30251717	Pooly conserved	no	Intronic	NCKAP5L

99	bta-mir-2428	bta-mir-2428	5	+	41964062	41964126	Pooly conserved	no	intronic	KIF21A
100	bta-miR-24-3p	bta-mir-24-2	7	-	12981642	12981713	Highly conserved	yes	intergenic	
101	bta-miR-2455	bta-mir-2455	7	-	17935950	17936025	Pooly conserved	no	intergenic	
102	bta-miR-2474	bta-mir-2474	8	-	62600141	62600199	Pooly conserved	no	Intronic	SHB
103	bta-miR-2478	bta-mir-2478	9	-	28046280	28046356	Pooly conserved	no	intergenic	
104	bta-miR-2483	bta-mir-2483	X	-	9189806	9189885	Pooly conserved	no	intergenic	
105	bta-miR-25	bta-mir-25	25	+	36892449	36892532	Highly conserved	yes	intronic	MCM7
106	bta-miR-26a	bta-mir-26a-1	22	+	11457900	11457989	Highly conserved	no	Intronic	CTDSPL
107		bta-mir-26a-2	5	+	55977923	55978006	Highly conserved	no	Intronic	CTDSP2
108	bta-miR-26b	bta-mir-26b	2	+	107133394	107133478	Highly conserved	no	intronic	CTDSP1
109	bta-miR-27a-3p	bta-mir-27a	7	-	12981791	12981868	Highly conserved	yes	intergenic	
110	bta-miR-27b	bta-mir-27b	8	+	83009823	83009919	Highly conserved	yes	intergenic	
111	bta-miR-28	bta-mir-28	1	-	79250531	79250616	Conserved	no	intronic	LPP
112	bta-miR-2881	bta-mir-2881	7	+	13754307	13754377	Pooly conserved	no	intergenic	
113	bta-mir-2882	bta-mir-2882	7	+	16664593	16664650	Pooly conserved	no	exonic	SMARCA4
114	bta-mir-2885	bta-mir-2885	29	-	40742016	40742073	Pooly conserved	no	intergenic	
115	bta-miR-2888	bta-mir-2888-1	21	+	36134507	36134571	Pooly conserved	no	intergenic	
116		bta-mir-2888-2	22	+	58391421	58391488	Pooly conserved	no	intronic	GRIP2
117	bta-miR-2892	bta-mir-2892	12	-	81323148	81323251	Pooly conserved	no	intergenic	
118	bta-miR-2893	bta-mir-2893	17	+	73346080	73346140	Pooly conserved	no	intronic	CABIN1
119	bta-mir-2898	bta-mir-2898	8	+	74353978	74354054	Pooly conserved	no	intergenic	
120	bta-miR-299	bta-mir-299	21	+	67563570	67563632	Pooly conserved	yes	intergenic	
121	bta-miR-29a	bta-mir-29a	4	-	95402319	95402382	Pooly conserved	yes	intergenic	
122	bta-miR-29b	bta-mir-29b-1	4	-	95402694	95402774	Highly conserved	yes	intergenic	
123		bta-mir-29b-2	16	+	77478033	77478113	Highly conserved	yes	intronic	LOC100848691
124	bta-miR-29c	bta-mir-29c	16	+	77478593	77478680	Highly conserved	yes	intronic	LOC100848691
125	bta-miR-30a-5p	bta-mir-30a	9	-	10768281	10768347	Highly conserved	no	intergenic	
126	bta-miR-30b-5p	bta-mir-30b	14	+	8084721	8084808	Highly conserved	yes	intergenic	
127	bta-miR-30c	bta-mir-30c	3	-	106059358	106059462	Highly conserved	yes	intronic	NFYC
128	bta-miR-30d	bta-mir-30d	14	+	8080292	8080361	Highly conserved	yes	intergenic	
129	bta-miR-30f	bta-mir-30f	9	-	10730929	10731012	Highly conserved	no	intergenic	
130	bta-miR-32	bta-mir-32	8	-	100305160	100305229	Highly conserved	no	intronic	TMEM245
131	bta-miR-320a	bta-mir-320a-1	8	-	70060384	70060465	Conserved	no	intergenic	
132		bta-mir-320a-2	20	+	15213924	15214005	Conserved	no	intergenic	
133	bta-miR-324	bta-mir-324	19	-	27571341	27571429	Conserved	no	intergenic	
134	bta-miR-331	bta-mir-331	5	+	25109057	25109146	Pooly conserved	no	intergenic	
135	bta-miR-338	bta-mir-338	19	+	52189629	52189720	Highly conserved	no	intronic	AATK
136	bta-miR-339	bta-mir-339a	25	+	42293998	42294075	Conserved	no	intronic	C7orf50
137	bta-miR-33a	bta-mir-33a	5	+	113410750	113410818	Highly conserved	no	intronic	SREBF2
138	bta-miR-342	bta-mir-342	21	+	66706848	66706941	Conserved	no	intronic	EVL
139	bta-miR-345-5p	bta-mir-345	21	+	66867086	66867176	Pooly conserved	no	intergenic	
140	bta-miR-34a	bta-mir-34a	16	+	45197390	45197496	Highly conserved	no	intergenic	
141	bta-miR-361	bta-mir-361	X	+	74328422	74328492	Conserved	no	intronic	CHM
142	bta-miR-362-5p	bta-mir-362	X	+	92898442	92898506	Pooly conserved	yes	intergenic	
143	bta-miR-365-3p	bta-mir-365-1	25	+	13344234	13344320	Highly conserved	yes	intergenic	
144		bta-mir-365-2	19	-	18811262	18811372	Highly conserved	yes	intergenic	
145	bta-miR-374a	bta-mir-374a	X	+	81951223	81951294	Conserved	yes	intergenic	
146	bta-miR-374b	bta-mir-374b	X	+	82023911	82024005	Conserved	yes	intergenic	
147	bta-miR-376b	bta-mir-376b	21	+	67579062	67579154	Conserved	yes	intergenic	
148	bta-miR-376d	bta-mir-376d	21	+	67578686	67578767	Conserved	yes	intergenic	

149	bta-miR-378	bta-mir-378-1	7	+	63067301	63067366	Conserved	no	intronic	PPARGC1B
150		bta-mir-378-2	4	+	10715298	10715373	Conserved	no	intergenic	
151	bta-miR-379	bta-mir-379	21	+	67561869	67561954	Conserved	yes	intergenic	
152	bta-miR-411a	bta-mir-411a	21	+	67563098	67563179	Pooly conserved	yes	intergenic	
153	bta-miR-423-5p	bta-mir-423	19	+	21799484	21799577	Pooly conserved	no	intronic	NSRP1
154	bta-miR-425-5p	bta-mir-425	22	+	51543952	51544038	Pooly conserved	yes	intergenic	
155	bta-miR-424	bta-mir-424	X	-	18185439	18185534	Highly conserved	yes	intronic	LOC100847497
156	bta-miR-450a	bta-mir-450a-1	X	-	18179070	18179151	Conserved	yes	intergenic	
157		bta-mir-450a-2	X	-	18179221	18179312	Conserved	yes	intergenic	
158	bta-miR-451	bta-mir-451	19	-	20796860	20796930	Highly conserved	yes	intergenic	
159	bta-miR-452	bta-mir-452	X	-	34665624	34665708	Pooly conserved	yes	intronic	GABRE
160	bta-miR-486	bta-mir-486	27	-	36261818	36261941	Conserved	no	intronic	ANK1
161	bta-miR-487b	bta-mir-487b	21	+	67583558	67583641	Conserved	yes	intergenic	
162	bta-miR-494	bta-mir-494	21	+	67569676	67569760	Conserved	yes	intergenic	
163	bta-miR-497	bta-mir-497	19	-	27441652	27441763	Highly conserved	yes	intergenic	
164	bta-miR-500	bta-mir-500	X	+	92899698	92899781	Pooly conserved	yes	intergenic	
165	bta-miR-532	bta-mir-532	X	+	92886501	92886586	Pooly conserved	yes	intergenic	
166	bta-miR-671	bta-mir-671	4	+	114614902	114615019	Pooly conserved	no	intronic	CHPF2
167	bta-miR-885	bta-mir-885	22	-	55072939	55073012	Pooly conserved	no	intronic	ATP2B2
168	bta-miR-92	bta-mir-92a-1	12	+	66227255	66227332	Highly conserved	yes	intergenic	
169		bta-mir-92a-2	X	-	17917857	17917924	Highly conserved	yes	intergenic	
170	bta-miR-93	bta-mir-93	25	+	36892249	36892325	Highly conserved	yes	intronic	MCM7
171	bta-miR-98	bta-mir-98	X	-	96382628	96382746	Highly conserved	yes	intronic	HUWE1
172	bta-miR-99a	bta-mir-99a	1	-	19931185	19931265	Highly conserved	yes	intergenic	
173	bta-miR-99b	bta-mir-99b	18	+	58014868	58014937	Highly conserved	yes	intergenic	

Chr. Chromosome, S. Strand,

Table 4.A2. Primer sequences for qRT-PCR

Gene	Description	Direction	Sequence 5' - 3'
ACTB	Beta-actin	Forward	CTAGGCACCAGGGCGTAA
		Reverse	CCACACGGAGCTCGTTGTA
EP300	E1A binding protein p300	Forward	GCCTTCGAAGAGATCGATGGT
		Reverse	TACTCTCCTCTGGTTGGGCG
PPARGC1B	peroxisome proliferator-activated receptor gamma, coactivator 1 beta	Forward	CGGCCTTGTGTCAAGATGGA
		Reverse	AGGTGAGGTGCTTGTGTAGC
PTK2	protein tyrosine kinase 2	Forward	CCAAATGGAGCCAGTGAACCT
		Reverse	AAGCACGTGGCCTGCTATG
SREBF2	sterol regulatory element binding transcription factor 2	Forward	GGGAGACCTACCACGCATC
		Reverse	GCTTCATGCAGGAACACCTT

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5 Effect of Age on bovine subcutaneous fat proteome: molecular mechanisms of physiological variations during beef cattle growth [8]

5.1 INTRODUCTION

Adipose tissue has significant influence on meat quality and animal productivity (Hausman et al., 2009; Dodson et al., 2010). It is also a dynamic organ as it is the main form of energy storage in mammals and performs important endocrine roles such as production of the hormone leptin (Galic et al., 2010). Understanding adipogenesis is a crucial step to develop strategies to manipulate adiposity in beef in an effort to produce even healthier meat products for consumers. Current knowledge of the molecular mechanisms controlling adipogenesis in bovine and other species arises mainly from transcriptomic studies (Basu et al., 2012). However, mRNA levels may not fully represent phenotype responses as transcript levels do not always correlate with protein expression (Schwanhausser et al., 2011). Therefore, the characterization of translated proteins may be a better tool to study adipose tissue physiology and metabolism. Several factors can influence adipogenesis and fat development including genetics, diet, fat depot location and age (Kirkland et al., 2002; Hosooka et al., 2008; Jin et al., 2012). Studies in beef cattle have shown intense

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developmental changes (hyperplasia and hypertrophy) in adipose tissue during growth (Robelin, 1981; Cianzio et al., 1985). Age has also been shown to affect gene expression in bovine adipose tissue (Wang et al., 2009). To date, few studies have investigated the proteome of bovine adipose tissue (Rajesh et al., 2010; Zhao et al., 2010) and these studies did not examine the nature of proteomic changes in bovine adipose tissue in growing beef cattle. Therefore, the aim of this study was to analyze the proteomic changes in subcutaneous adipose tissue in 12 and 15-month-old steers in an effort to characterize the molecular mechanisms that control fat development in growing beef cattle.

5.2 MATERIAL AND METHODS

5.2.1 Animal study and sample collections

Eight 12-month-old British-continental steers were selected based on similar body weight (456 ± 20 kg). They were housed in individual pens at the Lethbridge Research Centre and offered the same finishing diet (Table 5.1) and water *ad libitum.* The diet was supplied until slaughter at about 15.5 months of age. Several performance measures were recorded throughout the experiment including body weight gain, feed intake, feed conversion and carcass traits including cutability and subcutaneous fat thickness which have been reported elsewhere (He et al., 2012). Subcutaneous adipose tissue was collected from each steer through biopsy (0.2-0.5g of tissue) at 12 and 15 months of age. The 12-month-old biopsy was performed at 15 cm to the left of the last thoracic vertebrae of each steer. The 15-month-old biopsy was performed within the same area but 8

cm left of the scar from the initial biopsy. Subcutaneous fat samples were immediately frozen in liquid nitrogen, and kept at -80°C until further analysis. The animal study was approved by the Animal Care Committee of Lethbridge Research Centre, Agriculture Agri-food Canada with ACC# 0930.

Table 5.1 Diet formulation on a dry matter basis.

Feed Formulation	Percentage (%)
Barley grain, %	85.00
Barley silage, %	10.00
Vitamin & mineral supplement, %[1]	5.00

[1] Providing the following minerals and vitamin in 1 kg diet dry matter: 14.67 mg copper, 58.32 mg zinc, 26.73 mg manganese, 0.66 mg iodine, 0.23 mg cobalt, 0.29 mg selenium, 4825 IU vitamin A, 478 IU vitamin D and 32 IU vitamin E.

5.2.2 Measurement of adipocytes size

A portion of each above collected biopsy sample was placed in warm saline solution and transported to the laboratory and processed immediately after sampling for adipocyte size measurement, while a second portion of the sample was flash frozen in liquid nitrogen and used for protein extraction. Adipose tissues used for adipocyte measurement were cut into small pieces of approximately 80 mg and fixed with 1 mL of 5% osmium tetroxide (Cartwright, 1987). Then, samples were removed from the osmium tetroxide solution and placed in 8 M urea in physiologic saline (NaCl 0.9%) to soften the tissue and enable isolation of adipocytes. Adipocytes were washed with saline and transferred to a 24 well plate

for microphotography using an inverted microscope (Olympus CKX41, Olympus, Japan) with a digital camera (Moticam 2300, Motic China Group Co., Ltd., China). The diameter of adipocytes was determined by computer image analysis using software of Motic Images Plus 2.0 ML as described by He et al. (2010).

5.2.3 Protein extraction and total protein quantification

Adipose tissue samples stored at -80°C were ground using liquid nitrogen. Proteins were extracted from each sample by homogenizing 100 mg of ground adipose tissue with 1mL of 2-D protein extraction buffer-V with diluent II (Urea (< 8 M), Thiourea (< 5 M), and CHAPS (< 10%) (GE Healthcare, Uppsala, Sweden) added with DTT (40mM), using a Precellys®24 tissue homogenizer (Bertin Technologies, Saint Quentin, France). The homogenate was centrifuged at 17,000 x g for 30 min at 4°C and the supernatant was transferred to new tubes with care being taken to avoid the lipid layer formed. RC DC (reducing agent compatible and detergent compatible) protein assay based on the Lowry method (Bio-Rad, Hercules, CA, USA) was used to quantify total protein after extraction according to manufacturer's instructions to ensure that a minimum of 20 µg of protein was available for LC-MS/MS analysis.

5.2.4 Label free LC-MS/MS quantification

Total protein (30 µg) from each sample was subjected to label-free quantification LC-MS/MS (Old et al., 2005) at the Mass Spectrometry (MS) and Proteomics Resource of the W.M. Keck Foundation Biotechnology Resource

Laboratory, Yale University. Additional details on LC-MS/MS analysis are described in Online Supplement 1. Briefly, extracted proteins were precipitated using methanol/chloroform and dissolved in 8M urea/0.4M ammonium bicarbonate (pH=8.0) and DTT (45mM), incubated at 37°C for 20 min and cooled to room temperature. Following incubation with 100mM iodoacetamide (IAN) for 20 min at room temperature, the protein samples were digested with 3µg of Lys C overnight at 37°C, and then incubated with 3µg of trypsin for six hours at 37°C. LC-MS/MS was performed using a LTQ Orbitrap XL (Thermo Scientific, Waltham, MA, USA) equipped with a Waters nanoAcquity UPLC system and used a Waters Symmetry® C18 180µm x 20mm trap column and a 1.7 µm, 75 µm x 250 mm nanoAcquity™ UPLC™ column (35ºC) for peptide separation. A total of 0.2µg of each sample proteins was run and samples were randomized with 2 blanks after each run. Each sample was run in triplicate. LTQ Orbitrap XL acquired MS using 1 microscan, and a maximum injection time of 900 ms followed by three data dependant MS/MS acquisitions in the ion trap (Bordner et al., 2011) with 2.4 sec of total cycle time for both MS and MS/MS acquisition. Progenesis LC-MS software (Nonlinear Dynamics Ltd., New Castle, U.K) (www.nonlinear.com) was used for data analysis. All runs were selected for detection with an automatic detection limit. A normalization factor was then calculated for each run to account for differences in sample loads among injections. The MSMS were exported for Mascot database searching and results imported into the Progenesis LCMS software, where search hits were assigned to corresponding features.

5.2.5 Database search and protein identification

Proteins were identified by searching the .mgf files from Progenesis LCMS data analysis using Mascot search algorithm version 2.2.0. (Matrix Science Inc., London, U.K.) (Hirosawa et al., 1993). The data was searched using the Uniprot database (http://www.uniprot.org), bovine taxonomy with the following parameters: type of search (MS/MS Ion Search), enzyme (trypsin), variable modifications (carbamidomethyl (Cys), oxidation (Met)), mass values (monoisotopic), protein mass (unrestricted), peptide mass tolerance (± 20 ppm), fragment mass tolerance (± 0.6 Da), charge (+7), maximum missed cleavages (2), decoy (yes), and the instrument type (ESI-TRAP).

5.2.6 Bioinformatics analysis

Functional analyses for all proteins, differentially expressed (DE) up-regulated and DE down-regulated proteins were performed using Ingenuity Pathway Analysis Package (IPA) (Ingenuity® Systems, www.ingenuity.com). Molecules from the dataset were mapped to the Ingenuity Knowledge Base (http://www.ingenuity.com) and associated with molecular and cellular functions. right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set was relevant. A p-value < 0.05 indicated that the function was significant to the dataset. The Downstream Effects Analysis (IPA®) was based on proteins differentially expressed between 12 and 15 month old steers and determined if a biological function increased or decreased based on which proteins were involved along with

their expression values. Z-scores > 2 or < -2 indicated that the activity of a relevant function was significantly increased or decreased. Statements of significance for data analyzed through IPA in functional analysis (p-value) and downstream effect analysis (z-score) were calculated based on IPA algorithms.

5.2.7 Statistical analysis

In this study a matched pair design was used to compare the effects of growth using eight steers at two different ages (12 and 15 months old). Measurements of fat traits (adipocyte size and subcutaneous fat thickness) and protein expression for each age (12 and 15-month-old) were tested for normal distribution using Shapiro-Wilk test and equal variances using Levene's test. A two-sample paired t-test was used to compare means of fat traits ($p < 0.05$). Protein expression was considered differentially expressed between 12 and 15-month-old steers when $p < 0.05$ based on a two-sample paired t-test and a fold change > +1.2 or < -1.2. Correlations between individual measured fat traits (subcutaneous fat thickness and adipocyte size) and individual protein expression were calculated using Pearson correlation coefficient (R). Statistical analysis was performed with SAS software (SAS Inst. Inc., Cary, NC).

5.3 RESULTS

5.3.1 Adipose tissue changes with age

Evidence of development in subcutaneous adipose tissue of steers was found during the 3 months growth period between the 12 and 15 months of age. Measurements of subcutaneous fat thickness using ultrasound showed an increase

of more than 7 mm during this period ($p < 0.001$) (He et al., 2012) a 116% increase in thickness. Biopsy samples from subcutaneous fat revealed that hypertrophy of adipocytes also occurred between 12 and 15 months of age ($p = 0.026$) (Figure 5.1).

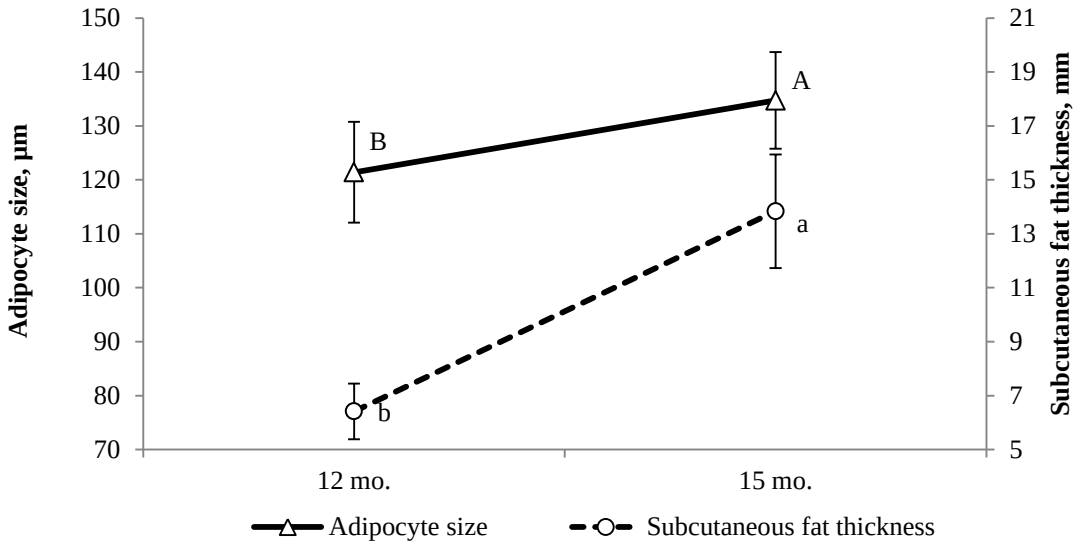

Figure 5.1 Subcutaneous fat thickness and adipocyte size over a three month growth period.

Different letters in adipocyte size means (A,B) and subcutaneous fat thickness means (a,b) indicate that they differ significantly ($p<0.05$).

5.3.2 Proteomic changes associated with age

A total of 627 proteins were identified and quantified in bovine subcutaneous adipose tissue using protein label free quantification. Almost all proteins were detected in both ages, with 626 identified at 12 months ($n = 626$) and all 627 being identified at 15 months. Expression of a total of 123 proteins changed ($p<0.05$) when 15 was compared to 12-months of age. The magnitude of

this change differed among proteins. For example, the heat shock 60kDa protein 1 varied less than 1.01 fold between 15 and 12 months while haptoglobin exhibited more than a 20 fold change. Difference in expression between 15 and 12 months was more than 5 fold for 11 proteins; 2-5 fold for 67 proteins, 1.5-2 for 109 proteins and 1.2-1.5 fold for 201 proteins. The remaining 239 proteins only varied up to 1.2 fold (Figure 5.2).

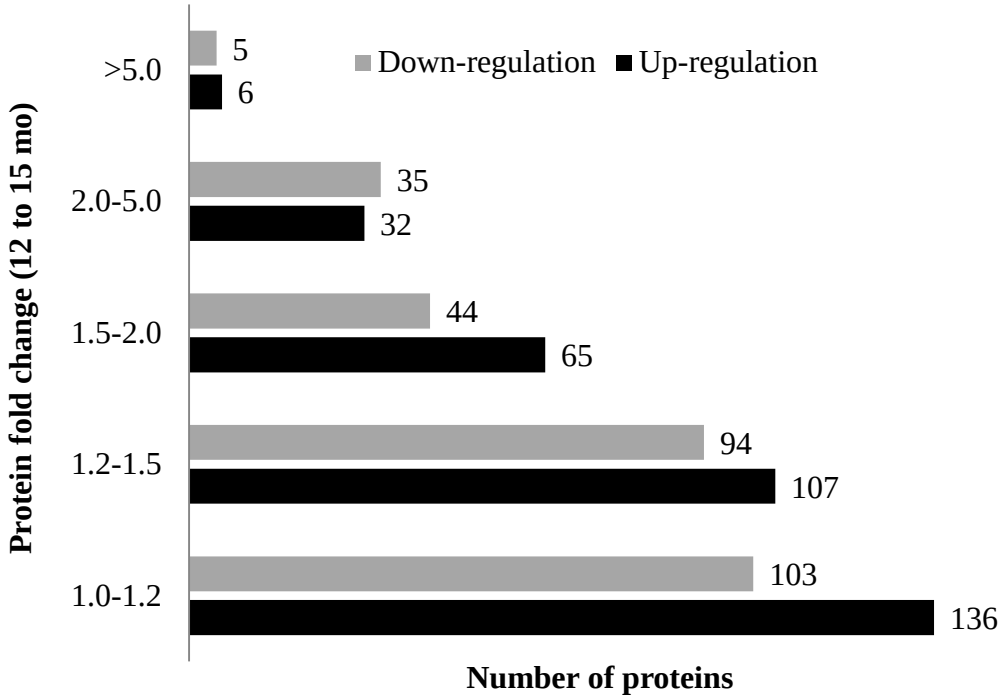

Figure 5.2 Changes in protein expression during growth (12 to 15 months old).

Despite being a uniform group of steers with similar body weight and age, the changes in protein levels were different according to each individual. For instance, steer #410 had 32.5% of proteins varying by more than 1.5 fold in expression between 12 and 15 months of age, while in the same period expression of 70.3% of proteins varied more than 1.5 fold in steer #211 (Figure 5.3). It is also

noticeable that the ratio between the amount of up-regulated and down-regulated proteins varied among steers. In three steers, most proteins were up-regulated by more than 1.5 fold between 12 and 15 months, #211 (63.0%), #307 (60.0%), and #405 (62.5%), while in five steers the number of down-regulated proteins were more abundant than those that were up-regulated, #108 (57.0%), #111 (53.5%), #209 (54.7%), #308 (61.4%), and #410 (68.6%) (Figure 5.3).

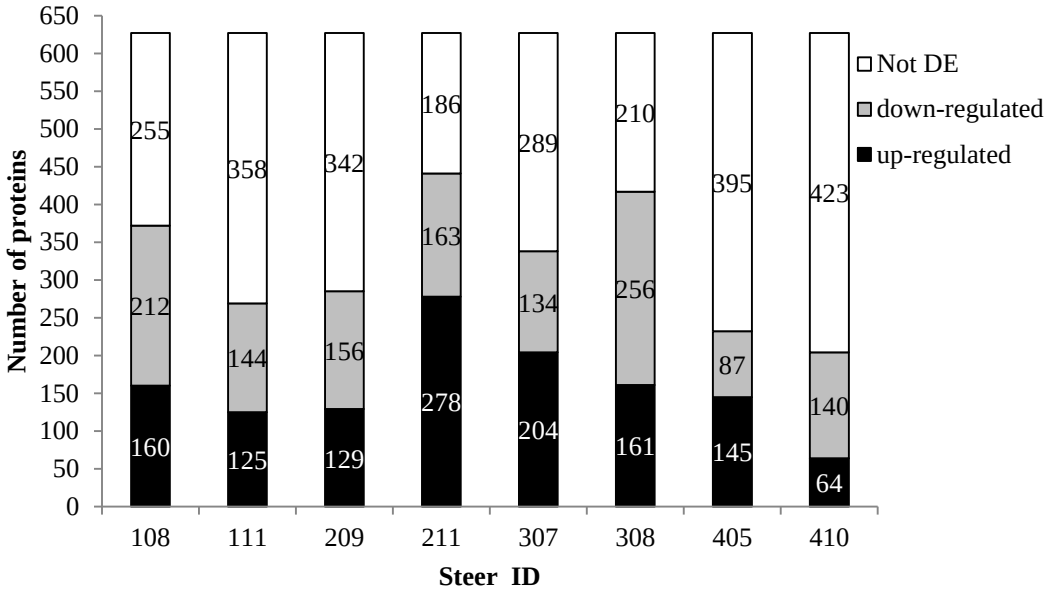

Figure 5.3 Quantity of proteins up or down-regulated more than 1.5 FC in individual steers.

Not DE represents proteins that were not differentially expressed (FC < 1.5) between 12 and 15 months of age

5.3.3 Relationship between protein expression and adipogenesis parameters

Taking into account that the steers displayed individual variation in protein expression and also possessed some variation in subcutaneous fat

thickness as well as adipocyte size, a correlation analysis was performed among the proteomic profiles of the eight steers and adiposity traits at 12 and 15 months of age. The top 10 proteins correlating with subcutaneous fat thickness or adipocyte size at both sampling points were selected and reported in Table 5.2. Several proteins that were highly correlated ($0.7 < R < -0.7$) with the adiposity traits were not included in the table as they did not exhibit this high correlation at both sampling points.

Table 5.2 Top 10 proteins correlated with thickness of subcutaneous fat or adipocyte size

Subcutaneous fat thickness				Adipocyte size			
Protein	**12 mo**	**15 mo**	**Average**	**Protein**	**12 mo**	**15 mo**	**Average**
ACLY	0.677*	0.590	0.634*	A2M	0.717**	0.701*	0.709**
FBP1	0.704*	0.523	0.613	HBA1	0.703*	0.548	0.626*
F13A1	0.658*	0.451	0.554	FNDC1	0.743**	0.503	0.623*
ARHGDIA	0.705*	0.355	0.530	HBB	0.641*	0.600	0.620
PRDX3	0.619	0.411	0.515	SPG20	0.841**	0.379	0.610
CLU	-0.616	-0.635*	-0.626*	RAB5C	-0.536	-0.895**	-0.715**
PLCH2	-0.924**	-0.339	-0.631*	TNS1	-0.590	-0.866**	-0.728**
SERPINA3	-0.611	-0.694*	-0.653*	LAMC1	-0.611	-0.875**	-0.743**
KRT78	-0.629*	-0.703*	-0.666*	PRKCDBP	-0.680*	-0.816**	-0.748**
KIAA1217	-0.626*	-0.739**	-0.682*	KCTD1	-0.733**	-0.845**	-0.789**

* $p < 0.1$ and ** $p < 0.05$.

alpha-2-macroglobulin (A2M), ATP citrate lyase (ACLY), Rho GDP dissociation inhibitor (GDI) alpha (ARHGDIA), clusterin (CLU), coagulation factor XIII, A1 polypeptide (F13A1), fructose-1,6-bisphosphatase1 (FBP1), fibronectin type III domain containing 1 (FNDC1), hemoglobin, alpha 1(HBA1), hemoglobin, beta (HBB), potassium channel tetramerization domain containing 1 (KCTD1), KIAA1217 (KIAA1217), keratin 78 (KRT78), laminin, gamma 1 (formerly LAMB2)(LAMC1), phospholipase C, eta 2 (PLCH2), peroxiredoxin 3 (PRDX3), protein kinase C, delta binding protein (PRKCDBP), RAB5C, member RAS oncogene family (RAB5C), serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 (SERPINA3), spastic paraplegia 20 (Troyer syndrome) (SPG20), and tensin 1 (TNS1)

5.3.4 Functions of proteins in adipose tissue during growth

A total of 627 UniProt accession numbers identified through label free quantification were mapped to 558 genes through Ingenuity Knowledge Base (IPA®). These genes were represented by two groups according to protein expression changes with a differentially expressed (DE) dataset consisting of 101 proteins ($p < 0.05$ and $FC > 1.2$) and a non-DE dataset consisting of 457 proteins ($p > 0.05$). Core analysis using IPA identified the most relevant biological functions for the total protein dataset. In total, 26 molecular and cellular functions were considered relevant ($p<0.05$) for proteins detected in bovine subcutaneous adipose tissue (Table 5.3). The functional analysis revealed that adipose tissue proteins had a broad spectrum of action ranging from basic functions such as cell death and survival, with 237 proteins associated to more specific roles such as protein degradation with only four proteins associated. All 26 relevant functions had at least one protein differentially expressed ($p<0.05$ and $FC >1.2$) between 12 and 15 months, suggestive of the widespread impact of growth on cellular metabolism.

Table 5.3 Differential expression of proteins in subcutaneous fat from steers at 12 to 15 months of age

Function	DE	%	Not-DE	%
Carbohydrate metabolism	7	10.9%	57	89.1%
Cell cycle	13	18.8%	56	81.2%
Cell death and survival	44	18.6%	193	81.4%
Cell morphology	31	19.3%	130	80.7%
Cell signaling	23	22.3%	80	77.7%
Cell-to-cell signaling and interaction	21	27.3%	56	72.7%
Cellular assembly and organization	31	20.3%	122	79.7%
Cellular compromise	4	15.4%	22	84.6%
Cellular development	22	17.1%	107	82.9%
Cellular function and maintenance	35	18.7%	152	81.3%
Cellular growth and proliferation	44	20.2%	174	79.8%
Cellular movement	39	23.2%	129	76.8%
DNA replication, recombination, and repair	7	15.9%	37	84.1%
Drug metabolism	1	6.7%	14	93.3%
Energy production	2	4.0%	48	96.0%
Free radical scavenging	6	10.5%	51	89.5%
Gene expression	7	16.3%	36	83.7%
Lipid metabolism	17	15.7%	91	84.3%
Molecular transport	27	16.4%	138	83.6%
Nucleic acid metabolism	3	5.6%	51	94.4%
Post-translational modification	2	12.5%	14	87.5%
Protein degradation	1	25.0%	3	75.0%
Protein folding	1	6.7%	14	93.3%
Protein synthesis	8	9.9%	73	90.1%
Small molecule biochemistry	20	13.2%	132	86.8%
Vitamin and mineral metabolism	10	24.4%	31	75.6%

DE represents proteins differentially expressed between 12 and 15 months of age ($p<0.05$), while Not-DE consists of proteins that did not differ statistically.

The set of proteins differentially expressed and mapped to the Ingenuity Knowledge Base (n=101) was further analysed by IPA Core Analysis as two separate sub-groups; up-regulated (n=58) and down-regulated proteins (n=43). Both up-regulated (Figure 5.4) and down-regulated proteins (Figure 5.5) were associated with a total of 25 functions (($p<0.05$) or –log (p-value) >1.3 which is used for a better graphical representation of significance). The top five functions for DE up-regulated proteins were cellular growth and proliferation, cell death and survival, cellular movement, cellular compromise, and cellular assembly and organization. Whereas the top five functions associated with DE proteins down-regulate were cellular movement, cell-to-cell signalling and interaction, cell death and survival, cellular function and maintenance, and cellular assembly and organization. A total of 22 functions were common to both DE up- and down-regulated proteins, with the order of functional relevance changing with regulation direction (Figures 5.4, 5.5). Functions in amino acid metabolism, energy production, and protein degradation were associated only with DE up-regulated proteins while gene expression, nucleic acid metabolism and RNA post-transcriptional modification were specific to DE down-regulated proteins. The prime function of adipose tissue, lipid metabolism, was relevant for both up-regulated and down-regulated protein datasets ($p < 0.001$) which illustrates the dynamic regulation of energy metabolism during growth.

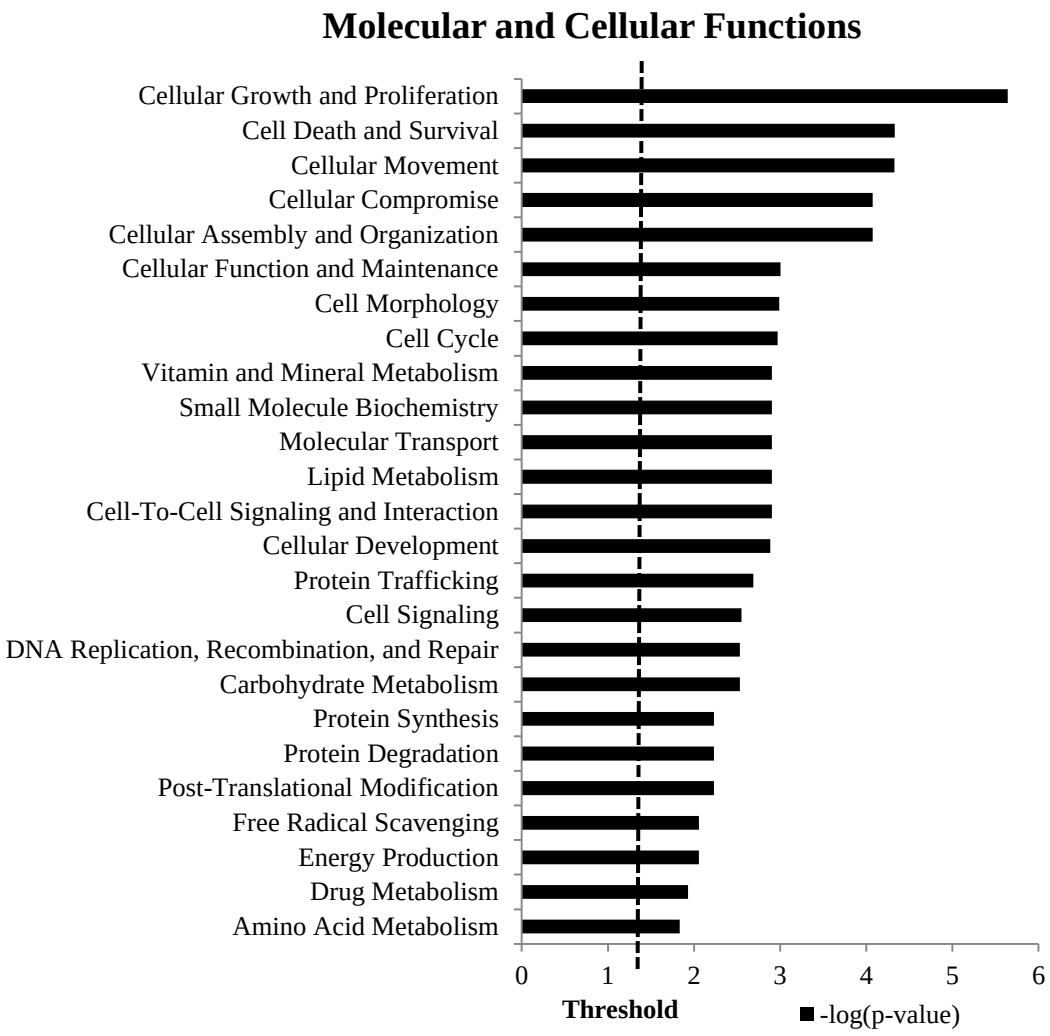

Figure 5.4 Molecular and cellular functions of proteins up-regulated in subcutaneous fat collected from steers at 12 and 15 months of age.

The likelihood of the association between the proteins in the dataset and a biological function is represented as –log(p-value), with large bars being more significant than short bars. The vertical line indicates the cutoff for significance (p-value of 0.05 or –log(p-value) of 1.3) which indicates that all functions displayed were significant.

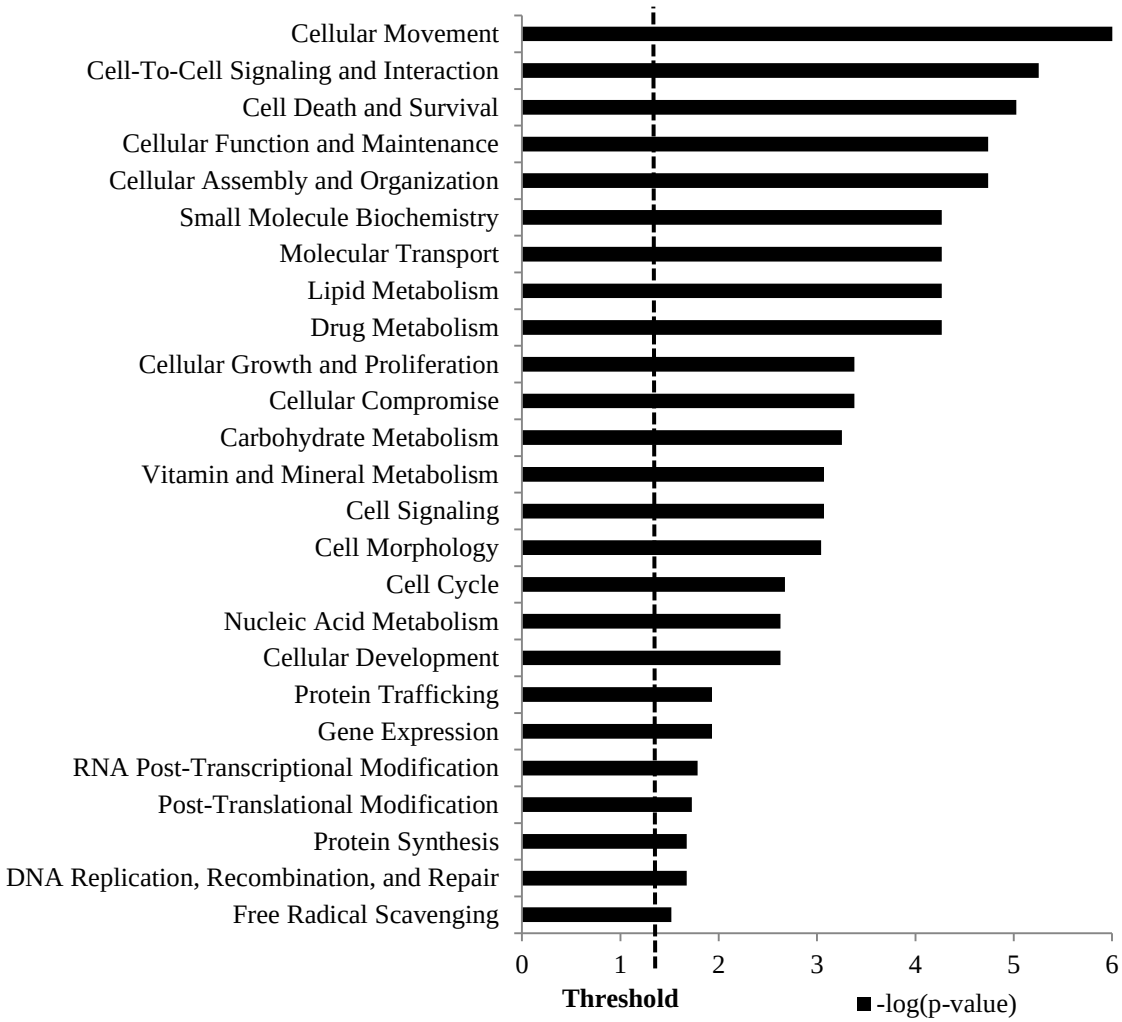

Figure 5.5 Molecular and cellular functions of proteins down-regulated in subcutaneous fat collected from steers at 12 and 15 months of age.

The likelihood of the association between the proteins in the dataset and a biological function is represented as –log(p-value), with larger bars being more significant than shorter bars. The vertical line indicates the cutoff for significance (p-value of 0.05 or –log(p-value) of 1.3) which indicates that all functions displayed were significant.

5.3.5 Lipid metabolism proteins

A total of 108 proteins expressed in subcutaneous adipose tissue were identified as molecules involved in different aspects of lipid metabolism by IPA Core Analysis. Among the functions identified, synthesis of fatty acids represented the main biological process in lipogenesis which supports the capability of adipose tissue to store energy in the form of lipids. A group of 26 out of 108 proteins were associated with the synthesis of fatty acids ($p = 6.73x10^{-6}$) (Figure 5.6). Half of these proteins (n=13) were known to increase fatty acid synthesis, five decrease it and eight proteins were involved in fatty acid synthesis but their exact function is presently unknown according to the Ingenuity knowledge Base®. Expression of four out of five proteins that inhibit fatty acid synthesis increased between 12 and 15 month samples, whereas 8 out of 13 proteins that promote fatty acid synthesis decreased (Figure 5.6).

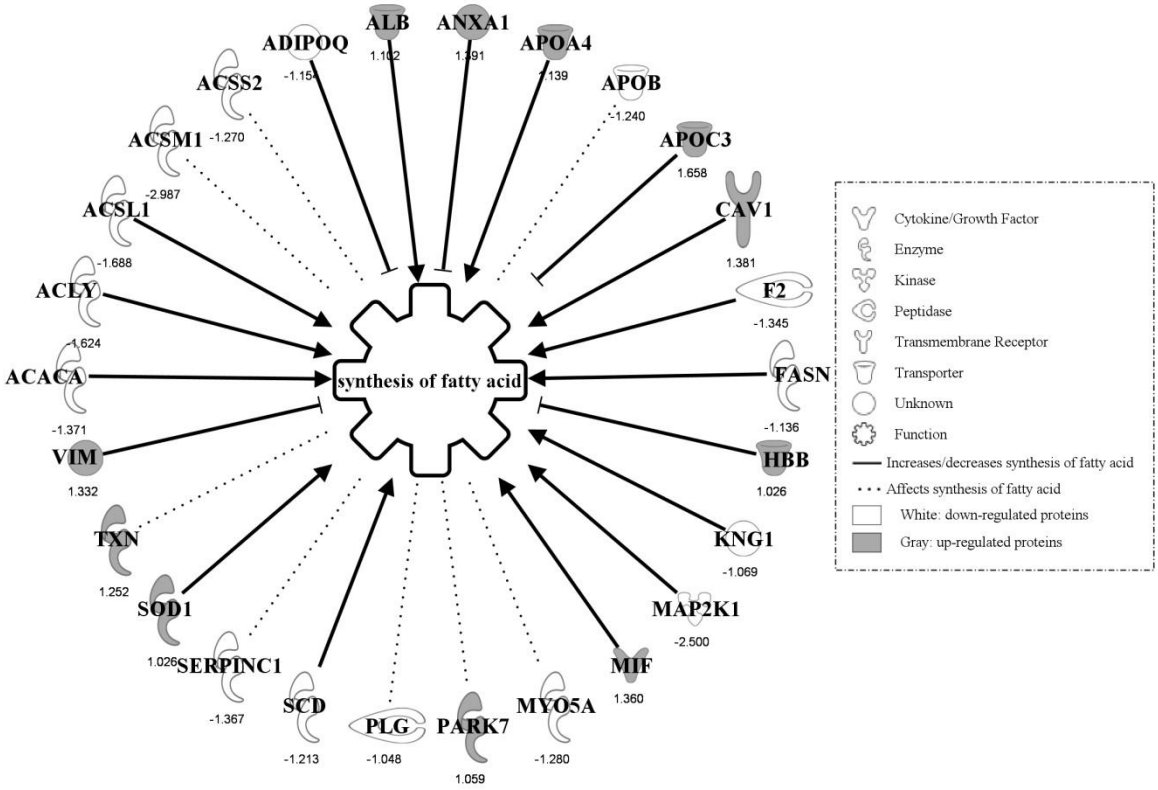

Figure 5.6 Effects of changes in the expression of proteins on the synthesis of fatty acids in steers at 12 and 15 months of age.

Numbers bellow the gene symbols represent the fold change between 12 and 15-month-old steers. acetyl-CoA carboxylase alpha (ACACA), ATP citrate lyase (ACLY), acyl-CoA synthetase long-chain family member 1 (ACSL1), acyl-CoA synthetase medium-chain family member 1 (ACSM1), acyl-CoA synthetase short-chain family member 2 (ACSS2), adiponectin, C1Q and collagen domain containing (ADIPOQ), albumin (ALB), annexin A1 (ANXA1), apolipoprotein A-IV (APOA4), apolipoprotein B (APOB), apolipoprotein C-III (APOC3), caveolin 1, caveolae protein, 22kDa (CAV1), coagulation factor II (thrombin) (F2), fatty acid synthase (FASN), hemoglobin, beta (HBB), kininogen 1 (KNG1), mitogen-activated protein kinase 1 (MAP2K1), macrophage migration inhibitory factor (glycosylation-inhibiting factor) (MIF), myosin VA (heavy chain 12, myoxin) (MYO5A), parkinson protein 7 (PARK7), plasminogen (PLG), stearoyl-CoA desaturase (delta-9-desaturase) (SCD), serpin peptidase inhibitor, clade C (antithrombin), member 1 (SERPINC1), superoxide dismutase 1, soluble (SOD1), thioredoxin (TXN), vimentin (VIM).

A set of 17 proteins were identified when the lipid metabolism proteins (n=108) were subjected to a 1.2 fold or greater change in expression ($p < 0.05$) in samples from steers at 12 and 15 months of age. Of these, ten proteins were up-regulated, from +1.323 to +1.910 fold and included annexin A1 (ANXA1), apolipoprotein C-III (APOC3), apolipoprotein H (beta-2-glycoprotein I) (APOH), EH-domain containing 1 (EHD1), gelsolin (GSN), lamin A/C (LMNA), myosin, heavy chain 9, non-muscle (MYH9), protein disulfide isomerase family A, member 3 (PDIA3), retinol binding protein 4, plasma (RBP4), and renin binding protein (RENBP). The other seven proteins were down-regulated with fold changes from -1.345 to -2.987 including acyl-CoA synthetase medium-chain family member 1 (ACSM1), coagulation factor II (thrombin) (F2), mitogen-activated protein kinase kinase 1 (MAP2K1), orosomucoid 1 (ORM1), flavoprotein (Fp) (SDHA), serpin peptidase inhibitor, clade C (antithrombin), member 1 (SERPINC1), and serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (SERPING1) in steers at 12 to 15 months of age (Table 5.4).

Table 5.4 Lipid metabolism proteins differentially expressed in steers at 12 and 15 months of age

Uniprot	Gene ID	Description	Expression 12mo	Expression 15mo	FC	p-value
Q2KIS1	RENBP	renin binding protein	$1.43x10^4±4.57x10^3$	$2.74x10^4±7.89x10^3$	1.910	0.015
P17690	APOH	apolipoprotein H (beta-2-glycoprotein I)	$3.91x10^4±1.89x10^4$	$7.43x10^4±3.24x10^4$	1.899	0.031
Q5E9R3	EHD1	EH-domain containing 1	$1.91x10^5±2.96x10^4$	$3.45x10^5±1.15x10^5$	1.812	0.005
P19035	APOC3	apolipoprotein C-III	$2.30x10^4±7.68x10^3$	$3.81x10^4±1.14x10^4$	1.658	0.027
P38657	PDIA3	protein disulfide isomerase family A, member 3	6.36x104±$1.64x10^4$	$9.47x10^4±3.17x10^4$	1.488	0.030
F1MYG5	LMNA	lamin A/C	$5.65x10^5±1.10x10^5$	$8.10x10^5±3.26x10^5$	1.433	0.048
F1N650	ANXA1	annexin A1	$5.41x10^5±1.87x10^5$	$7.53x10^5±1.67x10^5$	1.391	0.036
F1MJH1	GSN	gelsolin	$6.29x10^5±1.19x10^5$	$8.58x10^5±1.59x10^5$	1.364	0.008
F1MQ37	MYH9	myosin, heavy chain 9, non-muscle	$1.99x10^5±4.63x10^4$	$2.67x10^5±6.33x10^4$	1.340	0.017
G1K122	RBP4	retinol binding protein 4, plasma	$4.38x10^4±9.49x10^3$	$5.80x10^4±9.89x10^3$	1.323	0.015
P00735	F2	coagulation factor II (thrombin)	$4.78x10^4±9.54x10^3$	$3.55x10^4±1.04x10^4$	-1.345	0.011
F1MSZ6	SERPINC1	serpin peptidase inhibitor, clade C (antithrombin), member 1	$5.80x10^5±7.21x10^4$	$4.25x10^5±1.06x10^5$	-1.367	0.003
E1BMJ0	SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	$1.76x10^4±2.90x10^3$	$1.28x10^4±2.97x10^3$	-1.372	0.035
P31039	SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	7.91x103±$3.22x10^3$	$4.44x10^3±8.43x10^2$	-1.780	0.028
Q0VD16	MAP2K1	mitogen-activated protein kinase kinase 1	$3.61x10^5±9.56x10^4$	1.44x105±$3.20x10^4$	-2.500	<0.001
Q3SZR3	ORM1	orosomucoid 1	$3.67x10^6±2.10x10^6$	$1.41x10^6±6.37x10^5$	-2.596	0.006
F1MPP7	ACSM1	acyl-CoA synthetase medium-chain family member 1	$8.96x10^3±5.44x10^3$	$3.00x10^3±1.08x10^3$	-2.987	0.011

Fold change values refer to the average expression of 15 month old steers in comparison to 12 month old steers.

IPA core analysis was performed for the 17 lipid metabolism proteins differentially expressed in steers at 12- and 15-months of age (Table 5.4). The analysis revealed that these proteins were not only involved in lipid metabolism ($p = 8.45x10^{-11}$) but were also associated with up to 25 other functions, including carbohydrate metabolism (8 proteins, $p = 2.52x10^{-5}$) for example. Lipid metabolism possessed a total of 46 molecular and cellular sub-functions associated with the 17 DE proteins identified ($p < 0.05$). Proteins associated with 15 out of 46 lipid metabolism sub-functions involved in metabolic processes related to lipids, fatty acids and triacylglycerol are summarized in Table 5.5. The majority of proteins identified (10/17) were associated with fatty acid metabolism, while fewer proteins were found to be associated with other aspects of lipid metabolism such as transport of triacylglycerides (Table 5.5). The Downstream Effects Analysis (IPA®) revealed that there was a trend of reduced synthesis of lipids (z-score = -1.826) and reduced fatty acid synthesis (z-score = -1.836) in15-month-old as compared to 12-month-old steers, based on changes in the expression of ACSM1, ANXA1, APOC3, F2, MAP2K1, PDIA3 and SERPINC1. These results show that even though these 17 DE proteins represented only 15.7% of all proteins involved in the metabolism of lipids, they play a relevant and essential role in fat metabolism and development.

Table 5.5 Functions of lipid metabolism proteins differentially expressed in the subcutaneous fat from steers at 12 and 15 months of age

Functions	p-Value	Molecules
Fatty acid metabolism	$8.45x10^{-11}$	ACSM1 ↓, ANXA1 ↑, APOC3 ↑, APOH ↑, F2 ↓, GSN ↑, MAP2K1 ↓, RBP4 ↑, SDHA ↓, SERPINC1↓
Synthesis of fatty acid	$5.27x10^{-7}$	ACSM1 ↓, ANXA1 ↑, APOC3 ↑, F2 ↓, MAP2K1 ↓, SERPINC1 ↓
Transport of lipid	$6.15x10^{-7}$	APOC3 ↑, APOH ↑, F2 ↓, GSN ↑, RBP4 ↑
Synthesis of lipid	$8.95x10^{-6}$	ACSM1 ↓, ANXA1 ↑, APOC3 ↑, F2 ↓, MAP2K1 ↓, PDIA3 ↑, SERPINC1 ↓
Accumulation of lipid	$1.01x10^{-5}$	APOC3 ↑, F2 ↓, LMNA ↑, MYH9 ↑, RENBP ↑
Binding of lipid	$6.11x10^{-5}$	APOH ↑, F2 ↓, SERPING1 ↓
Redistribution of lipid	$2.06x10^{-4}$	APOC3 ↑, GSN ↑
Concentration of lipid	$2.33x10^{-4}$	APOC3 ↑, F2 ↓, LMNA ↑, MAP2K1 ↓, RBP4 ↑, SERPINC1 ↓
Homeostasis of lipid	$7.59x10^{-4}$	APOC3 ↑, EHD1 ↑, ORM1 ↓
Secretion of lipid	$8.44x10^{-4}$	ANXA1 ↑, APOC3 ↑, F2 ↓
Hydrolysis of lipid	$9.61x10^{-4}$	APOC3 ↑, F2 ↓, PDIA3 ↑
Release of lipid	$1.38x10^{-3}$	ANXA1 ↑, F2 ↓, SERPINC1 ↓
Metabolism of triacylglycerol	$3.58x10^{-3}$	APOC3 ↑, APOH ↑
Transport of triacylglycerol	$3.91x10^{-3}$	APOH ↑
Mobilization of triacylglycerol	$5.86x10^{-3}$	APOC3 ↑

The likelihood of the association between the proteins in the dataset and a biological function is represented as p-value, with lower p-values being more significant than larger p-values. The cutoff for significance was $p<0.05$.

↑ and ↓ arrows indicate up and down-regulation of proteins respectively.

5.4 DISCUSSION

Adipose tissue is a dynamic organ, as it stores energy in the form of lipids and it also plays an important endocrine role by regulating metabolism, with the release of leptin for example (Poulos et al., 2010). Understanding the molecular regulation of adipose tissue is a key step to improve beef quality, however the knowledge on how adipose tissue proteome is influenced by physiological factors is lacking. To the best of our knowledge, this study is the first to examine the effect of growth on bovine adipose tissue biology (*in vivo*) using a high throughput proteomic based approach. Our study characterized the molecular changes of adipose tissue of steers during growth at 12 and 15 months of age by profiling the expression of 627 proteins through label free quantification LC-MS/MS. It is worth noting that 627 proteins represent only a fraction of the total proteins expressed in bovine adipose tissue as 18,034 genes have previously been reported to be expressed at a single time in the subcutaneous fat of beef cattle (Jin et al., 2012).

Adipose tissue development relies on hypertrophy and hyperplasia of adipocytes and these processes are reported to occur more intensively when steers are young (Robelin, 1981; Cianzio et al., 1985). Increases in the thickness of subcutaneous fat and hypertrophy of adipocytes was observed in steers from 12 to 15 months of age (Figure 5.1). The change in adipocyte size represented an increase of about 11.6% in diameter, but considering that adipocytes are spherical shape, this represents a 36.6% increase in volume. The increase in adipocyte size alone (36.6%) is insufficient to account for the increase in subcutaneous fat

thickness and as a result cellular proliferation (hyperplasia) must have also played a role in fat deposition at this site. Other important changes in performance/adiposity traits have also been reported in these steers including increases in body weight from 454.1to 585.9 kg, rib eye area from 40.67 to 80.56 cm^2, and changes in the fatty acid profiles of subcutaneous fat (He et al., 2012). It is a common practice for the Canadian beef industry to finish the steers with a high grain diet for approximately 4 months, in order to reach a desired market weight and increase fat content, e.g. marbling. However, excessive fat deposition for example in subcutaneous or visceral fat is not desirable as much of that fat is not consumed reducing the animal productivity (Dodson et al., 2010). In this context, the age and diet of the steers in this study coincides with the finishing period of the beef industry.

Changes in the deposition of subcutaneous fat during beef cattle growth were also associated with modifications in the proteome profile within this tissue. There was more than a 1.5 fold change in the expression of approximately 28% of proteins in subcutaneous fat collected at 12 and 15 months (Figure 5.2). As the proteins originate from translation, similar changes are likely to occur at the level of mRNA as well. A study using microarray to measure gene expression of adipose tissue of mice at different ages (6, 14 and 18 months) observed that approximately 20% of the genes expressed were affected by age (Liu et al., 2011). Intramuscular fat collected from beef heifers at 3, 7, 12, 20, 25 and 30 months of age also displayed age related changes in gene expression, including that of

peroxisome proliferator activated receptor γ (PPARγ) (Wang et al., 2009) which is the main transcription factor regulating adipogenesis (Tang and Lane, 2012).

Surprisingly, the changes in proteome profile due to age were not uniform among steers (Figure 5.3). This individual variation may play an important role in the metabolism of adipose tissue in individual animals during growth resulting in differences in adipose deposition even though individuals were of similar age and fed similar diets. Genetics might have been a factor promoting individual variation as the steers originated from British-continental crosses from commercial operations in Alberta, Canada. Genetics has been shown to influence the adipose tissue molecular regulation. For example, Hereford × Aberdeen Angus vs Charolais × Red Angus steers were found to have 650 genes that were differentially expressed (> 1.5 fold) in subcutaneous adipose tissue (Jin et al., 2012). There is also evidence that genetics impacts microRNA expression in bovine adipose tissue (Jin et al., 2010). Due to individual variation in both proteomic expression and adiposity traits, a correlation analysis allowed the identification of proteins that were strongly positive or negative correlated with the thickness of subcutaneous fat and adipocyte size (Table 5.2). For example, the protein most positively correlated with subcutaneous fat thickness was ATP citrate lyase (ACLY) which is an enzyme located in the cytoplasm that produces acetyl-CoA, an essential building block for fatty acids biosynthesis in adipose tissue (Bauer et al., 2005). ACLY might represent a stable proteomic marker for subcutaneous fat thickness during the growing and finishing phase of beef cattle. Adipocyte size variations seemed to be associated with changes in the physical

structure of adipose tissue as three of the top proteins correlated to this variable: fibronectin type III domain containing 1 (FNDC1), laminin, gamma 1 (LAMC1) and tensin 1 (TNS1) were reported to be structural proteins (Gao et al., 2003; Saintigny et al., 2008; Yu et al., 2009).

The IPA core analysis revealed that both DE proteins and proteins not affected by age were involved in a myriad of molecular and cellular functions in bovine adipose tissue (Table 5.3). Proteins that were differentially expressed with age ($p<0.05$ and FC >1.2) accounted for a small proportion of the total proteins (101 vs. 457), yet they associated with all 26 functions identified within the complete protein dataset. Consequently, pinpointing the exact functional roles of this differentially expressed set of proteins is not feasible as they have a broad spectrum of activity within a cellular context, ranging from cell death and survival to post-translational modification.

The functional analyses focusing on proteins significantly up-regulated (Figure 5.4) or down-regulated (Figure 5.5) during growth showed that these age-related changes were not associated with specific molecular or cellular functions as the majority (88%) of the functions were identified in both up and down-regulated protein datasets. These findings illustrate the dynamic balance involving increases or decreases in protein expression which collectively regulate the complex biological functions of bovine adipose tissue. For example, the expansion of adipose tissue (hypertrophy and hyperplasia) can be clearly associated with the function cellular growth and proliferation which was assigned as the top function for the proteins up-regulated during growth; however, this

function was also relevant for the set of proteins down-regulated. Studies reported that proliferative and differentiation capabilities of adipocytes decrease in mice and rats as they age (Djian et al., 1983; Kirkland et al., 1990; Sepe et al., 2011) a response that is at least in part due to changes in expression of the adipogenic transcription factor CCAAT/enhancer binding protein α (C/EBPα) (Karagiannides et al., 2001). The steers in this study would still be considered young (15 months) as cattle can easily have a natural lifespan of more than 10 years. Evidence showing adipocyte hypertrophy and hyperplasia indicate that the proliferative and differentiation capacity was not reduced in bovines. Therefore, their adipocyte functions were not impaired as observed in mouse or rat studies which have analyzed the impact of age in individuals with a much shorter lifespan and more extreme age differences (young vs. old).

In ruminants, adipose tissue is the main anatomical site for lipogenesis as 92% of fatty acid synthesis occurs in this organ (Ingle et al., 1972), while in humans and birds the liver is the main site of synthesis and in rodents both liver and adipose tissue are relevant for fat synthesis (Nafikov and Beitz, 2007; Laliotis et al., 2010). Approximately 25% of the lipid metabolism proteins identified in this study were involved in fatty acid synthesis (Figure 5.6). Most of the proteins with known effects on fatty acid synthesis (12 out of 18) had expression changes suggestive of reduced lipogenesis in subcutaneous fat of steers at the end of finishing period (15 months). However, these results may not be representative of changes in protein expression that may occur in other fat depots (e.g visceral and intramuscular) as each location has distinct lipogenic activity (Ingle et al., 1972),

physiology (Palou et al., 2009; Rajesh et al., 2010) and develop in distinctive manners during aging (Cartwright et al., 2007).

Lipid metabolism did not rank as the top function for DE proteins up- or down-regulated during growth; however it was a relevant process for both datasets with expression of 17 proteins clearly changing in subcutaneous fat samples collected at 12 and 15 months of age (Table 5.4). The magnitude of change of DE proteins involved in lipid metabolism was not dramatic with a 1.3-3 fold change in expression. Although subcutaneous adipose tissue expanded considerably during 3 month period of growth, extreme changes in protein expression would not be expected as all individuals were in a positive energy balance, under the same environmental conditions, healthy and the interval of time represents a short duration relative to the overall lifespan of cattle. Even in mice, with a much shorter lifespan, the comparison of adipocytes between 6 and 14-month-old individuals have not shown extreme expression differences in the large majority of genes associated with adipocyte differentiation and other aspects of lipid metabolism (Liu et al., 2011). Other factors have been shown to have a larger influence on adipose tissue molecular regulation in comparison to age. For example, gene expression in adipocytes of rats varied by much as 8.4% across different fat depots (epididymal vs. perirenal fat) while this variation was only 0.02% in rats of different age (3, 17 and 30 month old) (Cartwright et al., 2010). In one of our previous studies using the same steers we observed that 51% of the proteins expressed differently due to different fat depot location (subcutaneous vs.

visceral fat) and 5.3% differed due to diet (high vs. low fat diet) (Romao et al., 2013).

DE proteins involved in lipid metabolism were associated with several metabolic processes including synthesis, transport and release of lipids (Table 5.5). Based on the results of the downstream effects analysis, the synthesis of lipids at the cellular level was reduced as the cattle aged. For example, proteins that have been reported to increase lipid synthesis were down-regulated at 15 months, such as MAP2K1 (Prusty et al., 2002). A previous study has shown that the lipogenic capabilities of bovine adipocytes from subcutaneous fat was dependent on the cell size and further observed that there is a decrease in the incorporation of acetate into lipids in large adipocytes (> 116 μm) isolated from finishing as compared to growing steers (He et al., 1998). During the same period, fat mass expanded in the steers as adipocyte size increased and subcutaneous fat levels almost doubled. The expression of ANXA1 was indicative of fat expansion in beef as it has already been reported to be associated with increased fat mass in mice (Warne et al., 2006) and increased subcutaneous fat thickness in beef cattle (Zhao et al., 2010).

In the face of these opposing results (reduction in lipid synthesis at the cellular level vs. increase in fat mass) it is possible that the overall synthesis of lipids by subcutaneous fat depot did not decrease from 12 to 15 months of age, as the reduction in lipid synthesis at the cellular level might have been compensated by the hyperplasia in adipose tissue. The mass of subcutaneous fat in steers was dependent on differences between lipid anabolism and catabolism, which

indicates that the continued combination of positive energy balance and growth led to an increased overall rate of fat deposition compared to fat degradation.

In conclusion, the period of finishing for beef cattle promoted a significant expansion of subcutaneous adipose tissue concomitant with proteomic expression changes associated with several biological processes, including lipid metabolism. Proteomic changes that occurred as the steers were growing indicated cellular growth and proliferation of adipose tissue but also a reduction in the synthesis of fatty acids at the cellular level. These findings suggested that the rate of fatty acids synthesis was adjusted during this growth period revealing a coordinated balance between subcutaneous fat mass and the cellular abundance of lipogenic proteins to regulate the rate of fat deposition in growing beef cattle. The results obtained in this study expand our understanding on the proteome dynamics of bovine adipose tissue during growth which might help the development in the future of new strategies to manipulate adiposity in beef cattle, improving meat quality and animals productivity.

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6 GENERAL DISCUSSION

6.1 MICRORNAOME IN BOVINE ADIPOSE TISSUE

MicroRNAs participate in several biological processes including adipogenesis (Alexander et al., 2011). Internal and external environmental factors can impact miRNA expression. In mice, changes in dietary fat (varying levels of conjugated linoleic acid in the diet) impacted the expression of several miRNAs (miR-143, -107, -221 and -222) from retroperitoneal adipose tissue (Parra et al., 2010). miRNAs levels have also been shown to differ among fat depots in humans (Rantalainen et al., 2011) and swine (Guo et al., 2012). Even the compositional structure of DNA that surrounds miRNA genes has been reported to affect how miRNAs function in humans (Yu et al., 2006). However the impact of conditions such as diet, fat depot and genomic context are not known on miRNAs in bovine adipose tissue. Therefore, we designed two experiments to analyze miRNA expression and function under different conditions using a high throughput miRNA profiling technique (miRNA microarray), qRT-PCR validation of transcripts and bioinformatics tools.

Microarray analysis in study 1 identified a total of 244 miRNAs and showed that most miRNAs were expressed in steers fed both diets. However, a parcel of miRNAs was specific to each diet which might tailor the regulation of adipose tissue to the specific metabolic requirements imposed by each dietary regime. These findings led us to further validate these miRNAs responsive to diet by qRT-PCR, confirming that eight miRNAs were diet sensitive (miR-19a, -92a, -92b, -101, -103, -106, -142-5p, and 296) and some of them have already been

shown to impact adipogenesis in other species such as miR-103 (Xie et al., 2009), -19a, and -92a (Wang et al., 2008). Besides, functional analysis of the predicted targets of these miRNAs revealed that they may play a role in several aspects of the regulation of lipid metabolism. Results from microarray analysis have also revealed that miRNAs are affected not only by diet but also by fat depot as their profile changed according to the type adipose tissue. We further validated through qRT-PCR the occurrence of depot specific miRNA expression, as miR-196a was highly expressed in visceral fat while miR-2454 was highly expressed in subcutaneous fat. Both miR-196a and miR-2454 were predicted to regulate genes involved in lipid metabolism. miRNA specificity in fat depots is likely to represent the need for different regulatory mechanisms to address the unique physiology of each tissue type.

Different dietary fat levels might not have been the only and/or direct influence on miRNA expression in bovine adipose tissue. The digestive physiology of ruminants can be affected by several factors such as feed intake, however there was no statistical difference between the two groups of steers in our study. Diet can also affect ruminant digestion, for example high levels of lipids in the diet can impact ruminal fermentation and consequently its products (Jenkins, 1993). The high fat and control diets differed in their fatty acids profiles as well. Although the process of biohydrogenation saturates most of the unsaturated fatty acids provided by a diet (Jenkins, 1993), there may be still a portion of unsaturated fat from the diet supplemented with flaxseeds that can escape biohydrogenation and can be utilized for lipid synthesis in adipose tissue.

Evidence shows that different fatty acids can have distinct effects on adipogenesis (Madsen et al., 2005). Therefore, future study on effect of animal intake and ruminal biohydrogenetaion under different diet need to be taken into account to clearly define the regulatory roles of miRNA in adipogenesis.

Most experiments exploring the roles of miRNAs in adipose tissue have aimed at identifying differential expression in individuals under contrasting physiological conditions. For example, using low fat vs. high fat diets (Chartoumpekis et al., 2012) or lean vs. obese individuals (Hurtado del Pozo et al., 2011) which reveals only particular aspects of miRNA biology in adipose tissue. Therefore, in study 3 we pursued a more holistic approach by exploring the association between miRNA global expression and function with the compositional structure of DNA that surrounds miRNA genes (genomic context features).

The results showed that the 155 miRNAs expressed in subcutaneous fat of all steers (AT core miRNAs) had genomic context features associated with their expression and function. For example, clustered miRNAs and highly conserved miRNAs were more highly expressed and had more predicted targets than non-clustered or less conserved miRNAs. A total of 34 miRNAs displayed coordinated behavior and were found to integrate six regulatory networks, which supported the idea that miRNAs may work collectively in a coordinated fashion to regulate gene expression in complex networks (Siengdee et al., 2013). Coordinated expression was also identified between two intronic miRNAs (miR-33a and miR-1281) and their host genes (*SREBF2* and *EP300*) reported to be involved in lipid

metabolism (Erickson et al., 2001; Horton, 2002). This suggested that these miRNAs regulate the lipid metabolism pathway that involves their host genes. miRNA roles are poorly understood, especially for those specific to a certain species. Almost 15% of the core adipose miRNAs in study 3 were bovine specific. Functional analysis of their gene targets revealed that 17 of them might be involved in aspects of adipose tissue metabolism unique to bovines or ruminants.

The results obtained in study 1 and 3 expand our understanding on miRNA function and behavior in bovine adipose tissue which may in the future help to identify new strategies to manipulate adiposity in beef cattle to improve meat quality and productivity.

6.2 PROTEOME IN BOVINE ADIPOSE TISSUE

The majority of studies that have explored the molecular regulation of adipose tissue have relied on transcriptomic data (Basu et al., 2012). Few attempts have been made to understand adipose tissue biology using a proteomics approach, particularly in beef cattle. Dietary change (Joseph et al., 2010), fat depot location (Bong et al., 2010) and age (Graugnard et al., 2009) have been shown to impact gene expression in bovine adipose tissue. However, mRNA and proteins have an overall poor correlation (Gry et al., 2009; Ghazalpour et al., 2011; Schwanhausser et al., 2011), which shows the importance of exploring the proteomics of bovine adipose tissue. Therefore, we performed two experiments using a high throughput method LC-MS/MS Label Free Quantification to profile protein expression. In study 2 proteome changes were analyzed in two fat depots

of steers fed different diets and in study 4 the proteomic changes were analyzed during growth in individuals at two different ages.

Changes in the fat content of diets (2.7% vs. 7.1%) led steers to present different adiposity phenotypes in subcutaneous fat, such as increased backfat thickness and adipocyte size. This dietary manipulation also promoted changes in proteomic expression of several proteins. However, it is worth mentioning that the proteomic changes observed due to diet may not be a sole effect on adiposity trait changes. A high fat diet might have increased adiposity traits in steers partly because the increased amount of dietary lipid (high fat diet) led to a higher amount of fatty acids to be absorbed in the intestine and eventually a higher availability for uptake by adipocytes which would increase in volume due to more triglycerides stored compared to steers fed a low fat diet. Different levels of dietary fat content influenced the expression of 36 out of 682 proteins detected in study 2, including six proteins involved in lipid metabolism (SCD, APOC3, ANXA6, PLIN1, MDH1, and ITGA6). The diet effect was more pronounced for subcutaneous fat as compared to visceral fat suggesting that fat depots respond differently to the inclusion of fat in the diet. This is relevant to beef quality, as visceral fat is discarded during processing, while subcutaneous fat is partially consumed with the muscles and seems to be more responsive to dietary changes. For instance, steers fed the high fat diet (flax supplemented) had higher levels of SCD which converts saturated fatty acids into monounsaturated fatty acids such as stearic acid into oleic acid, the most abundant fatty acid in bovine adipose tissue that also softens fat and impacts beef quality (Smith et al., 2006).

Distinct adipose depots have diverse physiological features in mammals (Baglioni et al., 2012). Our findings in study 2 revealed that approximately half of the proteins identified (51%) displayed expression changes according to the location of the fat depots. Studies have shown evidence of proteomic changes among fat depots using bovine adipose cell cultures (Rajesh et al., 2010) and human adipose tissues (Pérez-Pérez et al., 2009). Our results were further analyzed with a functional analysis that revealed that energy production and lipid metabolism were among the top functions associated with differentially expressed (DE) proteins. The distinct proteomic expression profiles were indicative of functional differences such as a higher synthesis and oxidation of lipids in visceral fat, which is in agreement with a proteomic study in humans (Pérez-Pérez et al., 2009). These findings suggest that the role of subcutaneous fat in beef cattle is more directed to energy storage while visceral fat is more active in lipid metabolism and energy balance.

miRNAs may have also played an important role in post transcriptional regulation as protein output from translation was not consistent with mRNA levels (R=0.245) for nine genes tested in study 2. Studies using more comprehensive datasets have also found that transcript levels do not necessarily correlate well with protein expression (Gry et al., 2009; Ghazalpour et al., 2011; Schwanhausser et al., 2011). Therefore, we used computational tools to predict which of the known bovine miRNAs could regulate the translation output of the proteins differentially expressed between subcutaneous and visceral fat with lipid metabolism functions. These filtered miRNAs were further compared to the

miRNAs detected in study 1 and we found out an overlap of 119 miRNAs. Eight of them were predicted to regulate more than 20% of the 38 DE lipid metabolism proteins, suggesting their importance to regulate bovine adipose tissue.

In study 4, we observed that during the finishing period (12-15months) subcutaneous adipose tissue of steers expanded with concomitant proteomic changes. The changes in protein expression were not uniform among individuals, which allowed us to perform correlation analysis between individual proteomic variations with individual fat traits measurements. That analysis revealed, for example, that ACLY which is an enzyme that produces acetyl-CoA, an essential molecule for fatty acids biosynthesis (Bauer et al., 2005) was positively correlated with backfat thickness at 12 and 15 months, being a potential protein marker. A total of 627 proteins were identified of which 123 were expressed differently between 12 and 15-month-old steers. A functional analysis showed that 20% of DE proteins were associated with cellular growth and proliferation, which supports the expansion of adipose tissue by hypertrophy and hyperplasia. Seventeen proteins were differentially expressed between ages and associated with different aspects of lipid metabolism (ACSM1, ANXA1, APOC3, APOH, EHD1, F2, GSN, LMNA, MAP2K1, MYH9, ORM1, PDIA3, RBP4, RENBP, SDHA, SERPINC1, and SERPING1). Downstream Effects Analysis predicted that the synthesis of lipids at cellular level was reduced as age increased based on the expression of DE lipid metabolism proteins. For example, MAP2K1 protein has been reported to increase lipid synthesis in adipocytes (Prusty et al., 2002) but was down-regulated when the steers got older. These results suggest that the rate

of cellular fatty acid synthesis was adjusted during the finishing period which reveals a coordinated balance between the amount of lipogenic proteins per cell and the subcutaneous fat mass, in order to regulate fat deposition during growth.

The proteomic studies (2 and 4) analyzed protein expression under different environmental (diets) and physiological (fat depot and age) conditions. These variables were highly distinct which led to different impacts on proteomic changes. We adopted two different criteria to filter DE proteins in studies 2 and 4 due to the different levels of intensity to which proteome changed in response to the variables studied. In study 2, a more stringent cutoff ($FC > 1.5$, $p < 0.01$) was employed to limit an exceeding number of DE proteins identified, especially due to fat depot effect. While in Study 4, age (animal growth) had a more discrete impact on protein expression changes in adipose tissue, therefore we decided to employ a less stringent cutoff ($FC > 1.2$, $p < 0.05$) in order to obtain an adequate set of DE proteins to perform bioinformatics analysis. The use multiple comparisons adjustment (e.g. false discovery rate) is commonly used in high throughput studies and can reduce the number of false effects identified but may also increase the number of true effects being missed. Therefore, we decided for a less conservative cutoff strategy in order to provide a more substantial number of proteins in DE datasets to allow a more in depth functional analysis of the proteomic data using IPA software.

The results obtained in this study 2 and 4 expanded our understanding on how bovine adipose is tissue molecular regulated. The proteomic changes observed in study 2 were supportive of the distinct metabolic and physiological

characteristics observed between subcutaneous and visceral adipose tissue depots in cattle. They also revealed how different depots respond to dietary changes. Besides, the study 4 characterized the proteome dynamics of bovine adipose tissue during growth. Understanding how fat deposition is regulated in beef cattle is crucial to improve meat quality (increase intramuscular fat or marbling) and improve productivity by reducing undesirable fat excesses, especially due to the higher energetic cost of depositing fat instead of muscle in the carcass. Our findings might help the development in the future of new strategies to manipulate adiposity in beef cattle, improving meat quality and animal productivity.

6.3 FUTURE DIRECTIONS

Although my work explored the molecular regulation of bovine adipose tissue using high throughput techniques, there were still some limitations. The characterization of miRNAs profile was performed using a customized bovine miRNA microarray. However, microarrays are able to detect only the expression of known miRNAs. At the time we designed the bovine miRNA microarray arrays, there were a total of 672 known bovine miRNAs (April 2010, miRBase v.15) and currently there are 783 miRNAs (June 2013, miRBase v.20). Therefore, the discovery of new miRNAs is continuous. Compared to other species such as humans (2578 miRNAs) or mice (1908 miRNAs), the bovine has much fewer miRNAs identified, showing that there is still work to be done in order to characterize fully the bovine microRNAome. In the face of this challenge, the use of next generation sequencing (miRNA-seq) could be a better alternative to

miRNA microarray. This technology has the capability of profiling not only the known bovine miRNAs, but also novel miRNAs and even their isoforms, which can add more information to the characterization of bovine adipose tissue microRNAome. Besides, next generation sequencing could also improve the accuracy of miRNA quantification. In our results we observed that several miRNAs had microarray expression levels divergent to those obtained by qRT-PCR. This fact also reinforces the need to perform qRT-PCR to validate miRNA expression levels from microarray experiments. Therefore, studies aiming to characterize and understand the biology of adipose tissue miRNAs could benefit greatly benefit from next generation sequencing.

In terms of data analysis, it is a common practice to use multiple comparisons adjustments such as false discovery rate (FDR) in microarray experiments. However, we did not use this step to filter miRNA responsive to diet or fat depot. Instead, we decided to further study (qRT-PCR and bioinformatics analysis) only miRNAs whose microarray results pointed to relatively high level of expression in bovine adipose tissue and had a depot or diet specific expression profile. This approach allowed us to focus on miRNAs that we thought had a higher biological interest to the context physiological differences in adipose tissue due to dietary changes and fat depot. Although our approach results are likely to overlap with those from an analysis including FDR, the later is a more conservative technique, which is likely to be more critical in larger experiments with the use of next generation sequencing.

Experiments with the purpose of profiling the adipose proteome are more challenging than those based on transcripts. In contrast to transcriptomic tools, proteomic technologies do not allow protein amplification therefore the detection of poorly expressed proteins is difficult. For example, important classes of proteins such as transcription factors were underrepresented in our studies. As a result, the coverage of molecules quantified in proteomics studies is less than in transcriptomic studies using microarrays or RNA-seq. In our experiment, we identified and quantified less than 700 proteins, however it is known that more than 18,000 genes are expressed at mRNA level in bovine adipose tissue (Jin et al., 2012). Besides, the extraction of proteins from tissues is less efficient than that of RNAs, as proteins have more complex and diverse biochemical structures than nucleic acids. Therefore, no extraction method is adequate for all proteins. In order to overcome this limitation in future studies, the use of a combination of extraction methods could be used to yield more proteins, instead of using only one general method of extraction.

The use of high throughput technologies to study the transcriptome and proteome of bovine adipose tissue can advance our understanding on how adipose tissue development and fat metabolism is molecularly regulated and help the development of strategies to manipulate adiposity and improving animals productivity and meat quality. However, these technologies must be matched with suitable experimental designs in which biological questions relevant to the beef industry could be answered. In this context, the use of individuals with diverse genetics (i.e. different breeds) representing distinct adiposity phenotypes (e.g.

high vs. low cutability) could provide an adequate resource to explore how genetics can impact transcriptomic and proteomic regulation of adipose tissue and consequently the phenotype. The effects of the interaction between genetics and environment on transcriptome and proteome of adipose tissue could also be explored by using individuals with distinct genetics, as mentioned previously, raised under different management practices (e.g. dietary manipulation). Therefore, valuable information could be provided that would explain how the beef industry practices interact with individuals with distinct genetics and what are the consequences of this interaction on the molecular regulation of bovine adipose tissue and phenotype.

In this study, subcutaneous and visceral adipose tissues were the only fat depots explored; however, other adipose locations are of great value to the beef industry, such as the intramuscular fat which is responsible for marbling, an important determinant of meat quality. The collection of intramuscular fat is more challenging because it is inside muscle tissue and needs to be carefully dissected from it to avoid contamination muscular cells. Therefore, efforts should be made to also include this fat depot in future studies analyzing the molecular regulation of different fat depots in beef cattle.

The dairy industry would also benefit from understanding how fat metabolism in adipose tissue is regulated. However, the objective would change from improving meat quality to increasing milk production. Therefore, studies exploring adipose tissue regulation and fat metabolism using dairy cow as subject are of great interest. Lactating cows rely significantly on lipid reserves on adipose

tissue to provide fatty acids to the milk during lactation, especially during negative energy balance. For example, during the first weeks of lactation, dairy cows producing an average of 30 Kg of milk/day were reported to secrete about 1.5Kg/day of lipids, of which 1 Kg/day was derived from adipose tissue reserves (Barber et al., 1997). Learning how adipose tissue is regulated during this physiological period could help the development of approaches to manipulate the adipose depots in order to improve milk production.

It is also important to consider that studying bovine adipose tissue *in vivo* results in a holistic view of the biology of fat development; however it does not easily allow the functional study of individual proteins and miRNAs of interest. Therefore, in future studies, the use of *in vitro* approaches represent an essential tool to manipulate the differentiation of adipose cells in controlled conditions. The specific functions and effects of miRNAs (e.g. miR-142-5p, miR-378, and miR-2474) in adipocyte cultures could be revealed inducing miRNA overexpression with miRNA mimics or promoting miRNA expression knockdown with RNA interference (RNAi) using anti-miRNA oligos. Similar approaches to overexpress or knockdown the expression of proteins (e.g. Annexin A1) based on their mRNAs could be applied to identify the functions of proteins. These strategy combined with downstream analyses (transcriptimics, proteomics, and metabolomics) could reveal their effects on adipocyte phenotype and molecular regulation, which can improve our understanding of their roles *in vivo* in ruminants.

6.4 IMPLICATIONS

This work has pioneered the exploration of the molecular regulation of beef cattle adipose tissue *in vivo* focusing on the microRNAome and proteome. The four experiments in this thesis were performed using novel profiling and bioinformatics techniques to analyze miRNA and proteins expression in adipose tissue of steers under different conditions. Our findings improved the understanding on how fat development is regulated in beef cattle. This knowledge may pave the road to the development in the future of strategies to manipulate bovine adiposity that consider the specific molecular regulation and physiology of different fat depot locations and their response to dietary manipulation, as well as the different periods of development of beef steers.

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