CAROLINA FILARDI DE CAMPOS

Gene Expression and Proteomic Analysis Underlying Adipogenesis in Livestock

Thesis submitted to the Breeding and Genetics Graduate Program of the Universidade Federal de Viçosa, in partial fulfillment of the requirements for the degree of *Doctor Scientiae.*

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RESUMO

CAMPOS, Carolina Filardi de, D.Sc., Universidade Federal de Viçosa, julho de 2016. **Expressão gênica e análise proteômica envolvidas na adipogênese em animais de produção.** Orientadora: Simone Eliza Facioni Guimarães.

Marmoreio ou gordura intramuscular (IMF) é um componente importante da produção animal, já que é um dos principais fatores para a qualidade de carne. IMF é alcançada pela adipogênese, processo de proliferação e diferenciação de pré-adipócitos, e pela lipogênese, com a assimilação subsequente de lipídeos. A compreensão dos eventos que ocorrem durante a diferenciação de pré-adipócitos tem avançado consideravelmente nos últimos anos e baseou-se principalmente no uso de cultura de tecidos. Os modelos animais podem ser utilizados não apenas para a melhor compreensão da deposição de gordura, mas também como modelos para maior compreensão sobre os mecanismos moleculares subjacentes a determinadas condições humanas. Além disso, a diferenciação em adipócitos tem muitas implicações para doenças em humanos. É sabido que esta diferenciação envolve uma cascata transcricional, de modo que o presente estudo proporciona conhecimento sobre genes e fatores de transcrição utilizando a técnica de RT-qPCR, envolvidos na diferenciação de pré-adipócitos em adipócitos maduros comparando duas raças divergentes de suínos. Os resultados mostram que alguns genes são diferencialmente expressos entre a linhagem comercial e a raça Piau. Além disso, um estudo de expressão gênica e um estudo proteômico são fornecidos revelando os efeitos da vitamina A sobre a adipogênese em bovinos, revelando efeito negativo sobre a adipogênese. Assim, destacamos a importância da expressão gênica e dos estudos proteômicos para aumentar a compreensão dos mecanismos moleculares envolvidos na adipogênese.

ABSTRACT

CAMPOS, Carolina Filardi de, D.Sc., Universidade Federal de Viçosa, July, 2016.
Gene Expression and Proteomic Analysis Underlying Adipogenesis in Livestock.
Adviser: Simone Eliza Facioni Guimarães.

Marbling or intramuscular fat (IMF) is an important component of livestock production, as it is a major factor in the overall meat quality. IMF is achieved by adipogenesis, the process of proliferation and differentiation of preadipocytes, and lipogenesis, with the subsequent assimilation of lipid. The understanding of events that occur during preadipocyte differentiation has advanced considerably in the last few years and has relied mainly on the use of tissue culture models of adipogenesis. Animal models can be used not only for a better understanding of fat deposition in livestock, but also as models to an increased comprehension on molecular mechanisms behind human conditions. Furthermore, adipocyte differentiation has many implications for human disease conditions. It is well known that this differentiation involves a transcriptional cascade, so the present study provided knowledge about genes and transcription factors using RT-qPCR technique, involved in differentiation of preadipocytes into mature adipocytes comparing two divergent pig breeds. The results show us that some genes are differentially expressed between commercial line and Piau breed. Moreover a gene expression and a proteomic study are provided revealing the effects of vitamin A on adipogenesis in cattle, revealing the negative effect on adipogenesis. Thereby, we highlighted the importance of gene expression and proteomic studies to increase the understanding of molecular mechanisms underlying adipogenesis.

CHAPTER I

GENERAL INTRODUCTION

Adipogenesis

Intramuscular fat (IMF) deposition is achieved at cellular level through adipogenesis, the process of proliferation and differentiation of preadipocytes, and lipogenesis, with the subsequent assimilation of lipid (Hausman et al., 2009; Dodson et al., 2010). Marbling or IMF is an important component of livestock production, as it is a major factor in the overall meat quality (flavor and tenderness), nutrition (protein and fat levels and fatty acid composition) and economic value (Hudson et al., 2015; Peña, et al., 2013; Sadkowski et al., 2014). IMF deposition involves a series of events that initiate and maintain preadipocyte proliferation, differentiation of preadipocyte into adipocytes and adipocyte maturation in combination with declining muscle growth (Sadkowski et al., 2014).

Many environmental factors can modulate how adipogenesis occurs. In live pigs, Serão et al. (2011) showed that, genes related to adipogenesis have different behaviors depending on the genetic background of the animal. In a more controlled analysis like *in vitro* studies have shown that cell culture conditions can induce differentiation of preadipocytes to adipocytes (Taxvig et al., 2013), such as adipogenic induction cocktails (Hausman et al., 2009; Peng et al., 2015), since adipocyte differentiation is under regulation of multiple hormones and growth factors (Hausman et al., 2009). Medium components can influence how cells will react, like Vitamin A, which has recently been shown to have a profound impact on all stages of adipogenesis and Vitamin D and folates

that interact with retinoic acid receptors to regulate adipogenesis (Wang et al., 2016). Regarding to *in vivo* studies, dietary additives have been cited as potential adipogenic/lipogenesis modulators in many studies, such as Zhong et al. (2011), who reported that dietary conjugated linoleic acids increase intramuscular fat content in pigs, and Ma et al. (2015) who observed that dietary arginine supplementation reduced stress levels, enhanced whole-body antioxidative function, and improved the meat quality of finishing pigs.

Adipogenic markers

Adipocytes hold the body's major energy reserve as triacylglycerols packaged in large lipid droplets. Adipocyte differentiation is controlled by many factors including genes and transcription factors (TFs) such as two principal adipogenic factors, CCAAT enhancer-binding protein alpha (*CEBPA*), and Peroxisome proliferator-activated receptor gamma (*PPARG*). PPARG is a member of the nuclear receptor superfamily of ligand-dependent transcription factors (TFs) and it is required for adipocyte differentiation, regulation of insulin sensitivity, lipogenesis, and adipocyte survival and function (Lefterova et al., 2014).

Gene expression studies

The understanding of events that occur during preadipocyte differentiation has advanced considerably in the last few years and has relied mainly on the use of tissue

culture models of adipogenesis (Ntambi and Young-Cheul, 2000). Furthermore, adipocyte differentiation has many implications for human disease status. It is well known that this differentiation involves a transcriptional cascade (Lefterova and Lazar, 2009), including gene and transcription factors, so in order to achieve a successful transformation into mature adipocytes, preadipocytes undergo marked changes in morphology and gene expression (Ali et al., 2013). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a rapid and sensitive method for gene expression measurement. Given the low amounts of mRNA in fat cells, qRT-PCR became the method of choice for gene expression studies in adipocytes (Arsenijevic et al., 2012).

Proteomic approach

Proteomics allows studying large numbers of proteins, including their post-translational modifications at a given point in time. Up to now, proteomic studies have been employed for relating one or two quality traits to a combination of potential biomarkers, trying to gain more knowledge on the physiology and biology of animal, muscle, cell or molecules (Gobert et al., 2014). One of the major goals of proteomics in the field of farm animal science is to shed light on skeletal muscle biochemistry (Picard et al., 2010) and to deepen our understanding of the physiological changes taking place at the protein level following harvest (D'alessandro and Zolla, 2013). Muscle growth and intrinsic properties of the muscle determine at least in part the quantity and quality of the

produced meat. Proteomics presents an interesting tool to increase our knowledge of muscle properties and how they develop during myogenesis (Picard et al., 2010).

Objectives and outliers

This thesis describes gene expression and proteomic studies in livestock animals regarding adipogenesis in muscle. In the second chapter, we aimed to present a review about animal models and the understanding of adipogenesis. Under this framework, we reported complex molecular processes of fat deposition in animals and summarized several issues regarding to Wagyu beef and other breeds related to intramuscular fat, since animals of this breed stand out for having higher marbling. The benefits of the use of Wagyu breed for adipogenic studies were also discussed. In the third chapter, we performed an experiment using stromal vascular pig cells obtained from fat samples, aiming to explain the processes involved in the commitment of adipogenic lineage and beginning of lipid accumulation by adipocytes comparing a commercial pig line and Piau local breed. To investigate genes and transcription factors involved in the adipogenesis process, we also aimed to build a combined genes-transcription factors network. In the fourth chapter, we present another study about gene expression in bovine cells involved in adipogenesis, aiming to identify the most important factors involved in differentiation of progenitor cells to adipocytes. Therefore, we performed an experiment looking for comparison of proteomic profiling of bovine skeletal muscle, influenced by the supplementation with vitamin A.

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CHAPTER II

REVIEW: ANIMAL MODEL AND THE CURRENT UNDERSTANDING OF MOLECULE DYNAMICS OF ADIPOGENESIS

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ABSTRACT

Among several potential animal models that can be used for adipogenic studies, Wagyu cattle is the one that presents unique molecular mechanisms underlying the deposit of substantial amounts of intramuscular fat. As such, this review is focused on current knowledge of such mechanisms related to adipose tissue deposition using Wagyu cattle as model. So abundant is the lipid accumulation in the skeletal muscles of these animals that in many cases, the muscle cross-sectional area appears more white (adipose tissue) than red (muscle fibers). This enhanced marbling accumulation is morphologically similar to that seen in numerous skeletal muscle dysfunctions, disease states and myopathies; this might indicate cross-similar mechanisms between such dysfunctions and fat deposition in Wagyu breed. Animal models can be used not only for a better understanding of fat deposition in livestock, but also as models to an increased comprehension on molecular mechanisms behind human conditions. This revision underlies some of the complex molecular processes of fat deposition in animals.

Keywords: wagyu, adipogenesis, lipodystrophies, obesity

RESUMO

Entre vários modelos animais potenciais que podem ser usados para estudos de adipogênese, a raça de bovinos Wagyu é aquela que apresenta os mecanismos moleculares subjacentes exclusivos no depósito de quantidades substanciais de gordura intramuscular. Assim, esta revisão é focada no conhecimento atual de tais mecanismos relacionados com a deposição de tecido adiposo usando a raça Wagyu como modelo. A acumulação de lipídeos nos músculos esqueléticos dos animais desta raça é tão abundante que, em muitos casos, a área de músculo transversal aparece mais branca (tecido adiposo) do que vermelha (fibras musculares). Esta acumulação aumentada de marmoreio é morfologicamente semelhante ao observado em inúmeras disfunções músculo-esqueléticas, doenças e miopatias; isso pode indicar mecanismos semelhantes entre essas disfunções e a deposição de gordura na raça Wagyu. Os modelos animais podem ser utilizados não apenas para uma melhor compreensão da deposição de gordura em animais de produção, mas também como modelos para uma maior compreensão sobre os mecanismos moleculares subjacentes às condições humanas. Esta revisão aborda alguns dos processos moleculares complexos na deposição de gordura em animais.

Palavras-chave: wagyu, adipogênese, lipodistrofias, obesidade

Introduction

Fat found in skeletal muscle includes intramyocellular fat droplets (occurring in lower abundance) and adipose tissue between muscle fibers, which is usually referred to as intramuscular fat (IMF). Adipose tissue is derived from the mesenchyme (Fehrer and Lepperdinger, 2005) with a supportive stroma (Romao et al., 2011) and is easily isolated (Hausman and Dodson, 2012) for *in vitro* studies. Adipocytes appear to be dynamic; a renewed search for the origin of adipocyte progenitors has demonstrated high incidences of cellular plasticity, even in adult adipose tissue (Fernyhough et al., 2005 and 2008; Hausman et al., 2009). As such, adipose tissue may represent a source of stem cells that can have far-reaching effects on several fields (Zuk et al., 2002). Furthermore, adipose tissue has the potential to be a source of cells for tissue engineer purposes, as it appears to contain cells able to act as functional and vascular building blocks for several tissues (Fraser et al., 2006). The potential for cellular development of adipocytes is believed to be fixed relatively early in life, with changes thereafter in either the size or number of cells that occur in proportion to the initial cell number and lipogenic proteins (Caserta et al., 2001; Pethick et al., 2004; Wang et al., 2009). Moreover, dysfunction of the adipose compartment (cells and metabolism) is central to the pathology associated with metabolic diseases such as obesity, type II diabetes (Edelman, 1998), cancer cachexia and lipodystrophies (Cristancho and Lazar, 2011).

Regarding to IMF in production animals, we can highlight the Wagyu beef cattle, which can be considered a model for the studies of adipose tissue deposition. This is a composite breed comprised of four Bos taurus breeds: Japanese Black, Japanese Brown, Japanese Shorthorn and Japanese Polled. Originally bred for strength and endurance, by

the 20th century, Wagyu cattle were mostly selected for their desirable marbling characteristics, with strict geographic restraints resulting in a sharp decline in genetic diversity (further amplified by artificial insemination), such that the offspring of five sires accounted for 42% registered Wagyu (Scraggs et al., 2014). The importation of Wagyu cattle to the United States in 1973 has allowed for the expansion of the population from the initial individuals in 1994 to an estimated 700 purebred animals in 2009 (Scraggs et al., 2014). Wagyu cattle are now highly valued for their tender meat (Yang et al., 1999) and their ability to deposit extremely high amounts of IMF (Fernyhough et al., 2008; Shirouchi et al., 2014). Comparisons between Wagyu cattle and other breeds regarding cell biology and general adipogenesis have been reported in several studies, such as those by Oikawa et al. (2000), Hausman et al. (2009), Dodson et al. (2010a) and Duarte et al. (2013). Some studies comparing Wagyu cattle with other breeds (Hausman et al., 2009; Dodson et al., 2010a; Duarte et al., 2013) have emphasized the uniqueness of this breed and the importance of better understanding adipogenesis, mainly because its increased accumulation of fat within the skeletal muscle, which seems to not follow the same pattern of other adipose tissue depots. Consequently, Wagyu cattle may present a highly marbled beef without an increase of overall fatness, making this breed an unique animal model for the understanding of this phenomena.

Adipogenesis and IMF

In humans, the subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) have been identified as the main fat depots. These depots differ in terms of their turnover. Adipose cells derived from VAT, in particular, have higher levels of metabolic

activity and have been shown to hypertrophy in obese individuals. In addition to VAT and SAT, intermuscular adipose tissue and IMF are also measured in farm animal species such as swine and cattle (Komolka et al., 2014).

IMF deposition is achieved at the cellular level through adipogenesis, the process of proliferation and differentiation of preadipocytes, and lipogenesis, the subsequent assimilation of lipid (Harper and Pethick, 2004; Hausman et al., 2009; Dodson et al., 2010b). Previous studies have suggested that the high deposition of IMF in Wagyu is related to the differential expression of specific genes (Hudson et al., 2014). In most cattle breeds, IMF is first visible at about 11 months, with the greatest increases between 15 and 24 months. However, when they are finished on concentrated grain, Wagyu deposit more IMF at an earlier age when compared with other cattle breeds (Wertz et al., 2002; Shirouchi et al., 2014). Dietary conditions, particularly energy density, affect carcass adipose tissue deposition in beef cattle (Yamada et al., 2009). Thus, cattle that are fed low energy through the finishing phase with forage and pasture type diets seldom show the desired marbling. Moreover, the level of dietary starch that is fed to young cattle may alter gene networks associated with adipocyte differentiation and energy metabolism (Graugnard et al., 2010). Thus, high energy diets (finishing diets usually only during the last 4 to 6 months before harvest) have been used as a production strategy for enhancing intramuscular adipose tissue in beef cattle. Exposure to high-starch diets during the early growth phase of cattle might induce precocious preadipocyte differentiation and lipid filling (Graugnard et al., 2010).

Marbling is an important component of livestock production, as it is a major factor in the overall meat quality (flavor and tenderness), nutrition (protein and fat levels

and fatty acid composition) and economic value (Pena et al., 2013; Hudson et al., 2014; Sadkowski et al., 2014). An increase in IMF has been proposed to occur either through the myogenic transdifferentiation of myogenic stem cells (MSC) in skeletal muscle through a complex regulatory pathway, which remains unclear, or through the multi-step process of adipogenic determination and differentiation of fibroblastlike preadipocytes into mature adipocytes assimilating lipid (Du and Zhu, 2010; Du et al., 2013).

Myoblasts and adipocytes have been shown to utilize intercellular communication, which directly affects the growth and development of these cells (Kokta et al., 2004; Muthuraman, 2014). Furthermore, when in close proximity, these cells perform a paracrine function by altering the growth, development or energy storage of each other based on the chemical factors that are released (Kokta et al., 2004; Muthuraman, 2014). Myoblasts have been shown to regulate the growth, development, differentiation and lipid assimilation of adipocytes through intercellular communication (Muthuraman, 2014).

Myostatin, a growth differentiation factor secreted by myoblasts, suppresses proliferation of preadipocytes in muscle tissue through direct down-regulation of adipogenic transcription factors, thus decreasing the overall IMF (Komolka et al., 2014). Furthermore, co-culture of myoblasts and adipocytes results in an upregulation of peroxisome proliferator activating receptor γ (*PPARγ*), CCAAT enhancer binding protein α (*C/EBPα*) and fatty acid binding protein 4 (*FABP4*), possibly indicating myoblasts' promotion of adipogenic specific transcription factors leading to increased IMF or allowing for MSC transdifferentiation as a mechanism for increasing IMF (Muthuraman, 2014). In both cases, myogenic and adipogenic contributions to marbling appear to be

mediated by the transcription factors, *PPARγ*, *C/EBPα* and *FABP4*. Fibroblasts like preadipocytes, in response to the aforementioned transcription factors, differentiate from progenitor cells and undergo adipogenesis and assimilation of lipid (Du et al., 2010). Similarly, MSCs respond to high concentrations of the same transcription factors. However, these factors appear to inhibit myogenic differentiation stimulating adipogenesis and also appear to inhibit myogenic differentiation, while ensuring adipogenic differentiation (Teboul et al., 1995; Taylor-Jones et al., 2002; Singh et al., 2007; Du et al., 2010). Considering this, the study of adipogenesis and its effects on the accumulation of IMF can be used not only to understand fat deposition in livestock, but also for a better comprehension of the differentiation process in mesenchymal stem cells.

Stem cells

Skeletal muscle is derived from the mesoderm and is postnatally surrounded by small multipotent myogenic satellite cells (SC) that play an important role in muscle hypertrophy and regeneration (Kook et al., 2006; Du et al., 2010; Lee et al., 2012; Duarte et al., 2014). SC are multipotent cells capable of transdifferentiating into intramuscular adipocytes when exposed to local cellular signaling (Taylor-Jones et al., 2002; Singh et al., 2007; Du et al., 2010; Lee et al., 2012; Ryan et al., 2013). Moreover, muscle side population (SP) cells are multipotent stem cells that can participate in myogenesis and muscle regeneration upon transplantation (Penton et al., 2013). Interestingly, SP cells in skeletal muscle tissue have not only the ability to transdifferentiate into cells from myogenic but also in hematopoietic lineage (Reecy et al., 2003). In vivo studies using mice as a molecular model, have confirmed the hematopoietic stem cell potential of SP

cells, as these present a regenerative capacity for blood, bone and lymph cells (Reecy et al., 2003). These studies have implied that SP cells may be an important resident source of transdifferentiation within skeletal muscle, and may be less differentiated than other previously discovered SC populations (Reecy et al., 2003). The regenerative capacity of skeletal muscle decreases with age, thereby resulting in an overall loss of muscle mass over time and an increase in lipid content (Teboul et al., 1995; Kook et al., 2006; Aguiari et al., 2008; Ryan et al., 2013). This might not be totally accountable by the decrease in stem cell activity; myogenic transdifferentiation into adipocytes also play a role in this phenomena. Since MSCs are multipotent, their transdifferentiation into intramuscular adipocytes is feasible, depending on different cellular signaling exposure, thereby leading to greater amounts of IMF (if the adipocytes invade the perimysium) (Taylor-Jones et al., 2002; Singh et al., 2007; Aguiari et al., 2008; Du et al., 2010; Lee et al., 2012).

Adipogenic markers

A major marker of adipogenesis (or lack thereof) is preadipocyte factor-1 (pref-1), as this membrane protein is expressed on preadipocytes and acts to prevent adipogenesis. It has been reported that pref-1 inhibits adipocyte differentiation via sex determining region Y-box 9, which binds to its binding sites at the *C/EBP* β and δ promoter regions to suppress their transcription (Sul, 2009). In addition, it appears to be depot specific, as high levels of pref-1 expression were observed in smaller adipocytes (Yamada et al., 2014). Early and late differentiation are marked by the expression of the C/EBP family, which in turn is greater in fat depots of Wagyu when compared with Holstein cattle, as they are responsible for the increased proliferation potential of Wagyu preadipocytes in

vitro (Yamada et al., 2009). Meanwhile, PPARs act as transcription factors to regulate gene expression by acting on lipid metabolism and adipocyte filling. Indeed, both *C/EBPα* and *PPARγ* from the IMF of Wagyu are increased when compared with the IMF of Angus cattle (May et al., 1994; Yamada et al., 2007; Duarte et al., 2013).

Previous studies have suggested that the high deposition of IMF in Wagyu cattle is related to the differential expression of specific genes such as *C/EBPα* and *PPARγ* (late adipogenic markers) as well as an early adipogenic marker zinc finger protein 423 (Duarte et al., 2013). Table 1 shows the differences in adipogenic gene expression between Wagyu and other cattle breeds. Moreover, the expression of adipogenesis and the lipid droplet associated genes, perilipin 1 (*PLIN1*) and adipose differentiation-related protein (*ADFP*), are upregulated as markers of the overall amount of IMF (Shirouchi et al., 2014). *PLIN1* is a major protein that resides on the surface of mature adipocyte lipid droplets and plays an integral role in triacylglycerol storage and breakdown (Shirouchi et al., 2014). The largest lipid droplets in mature adipocytes are exclusively coated with PLIN1. Moreover, *PLIN1* promotes skeletal muscle lipid deposition by partitioning excess fatty acids towards triacylglycerol storage (Shirouchi et al., 2014). *ADFP* functions similarly to *PLIN1* but it is ubiquitous, whereas *PLIN1* appears to be found only on adipocytes (Shirouchi et al., 2014).

Several genes are well known for their correlation with obesity and other metabolic disorders; these genes include *PPARγ* (Dodson et al., 2010b), leptin (*LEP*) (Duarte et al., 2007), adiponectin (*ADIPOQ*), *FABP4* (Wang et al., 2005), bone morphogenetic protein 4 (*BMP4*) (Majka et al., 2011), fat mass and obesity associated (*FTO*) (Fischer et al., 2009), *C/EBPβ* (Cristancho and Lazar, 2011) and v-akt murine

thymoma viral oncogene homolog 2 (*AKT2*). Three of the genes that are reported above (*BMP4*, *C/EBPβ* and *AKT2*) are found in many reports related to fat storage in humans. Five of them (*PPARγ*, *FABP4*, *ADIPOQ*, *FTO* and *LEP*) are mentioned both in connection with human disorders and adipogenesis in B. taurus. In particular, the role of the *ADIPOQ* gene is well described in Wagyu cattle (Jordan et al., 2011).

Adipogenesis and lipid metabolism: Wagyu as a model

Although researches in humans and farm animals ultimately have different goals – identification of potential drug targets for metabolic diseases v. optimization of meat quality – the same tissue can be the focus of both research efforts (Komolka et al., 2014). Bovine adipocytes are a cell model for studying adipogenesis and lipid metabolism for improving animal production and also serving human health (Duarte et al., 2013).

Table 1. Differences of adipogenic gene expression between Wagyu and other cattle breeds

Tissue / Cell type	Gene name	Variation	Reference
Sternomandibularis muscle tissue	*C/EBPα*, *PPARγ*, *Zfp423*	The mRNA expression of C/EBPα, PPARγ, and Zfp423 in Wagyu was higher than that of Angus at 24 months.	(Duarte *et al.*, 2013)
Intermuscular fat tissue	*C/EBPδ*	The mRNA expression of C/EBPδ was higher than that of Holstein at 19 months.	(Yamada *et al.*, 2009)
Mesenteric fat tissue	*C/EBP* family	The mRNA expression of C/EBP family was higher in Wagyu than that of Holstein at 19 months.	(Yamada *et al.*, 2009)
	Pref-1	The mRNA expression of the pref-1 gene was lower	(Yamada *et al.*, 2014)

		in Wagyu than that of Holstein at 19-24 months.	
Subcutaneous fat tissue	*PPARγ*	The protein expression of PPARγ in Wagyu was lower compared with that of Angus at 12 months.	(Wei *et al.*, 2015)
	C/EBPδ	The mRNA expression of C/EBPδ was higher in Wagyu than that of Holstein at 19 months.	(Yamada *et al.*, 2009)
	FABP4	The mRNA expression of FABP4 was lower in Wagyu compared with that of Holstein.	(Albrecht *et al.*, 2011)
Subcutaneous fat derived stromal vascular cells	PPARγ	The mRNA expression of PPARγ was lower in Wagyu when compared with that of Angus at 12 months.	(Wei *et al.*, 2015)
	TGFB3, *BMP2*	The mRNA expression of TGFB3 and BMP2 in Wagyu was lower than that of Angus at 12 months.	(Wei *et al.*, 2015)

C/EBPα = CCAAT enhancer binding protein α; *PPARγ* = peroxisome proliferator activating receptor γ; *Zfp423* = zinc finger protein 423; *Pref-1* = preadipocyte factor-1; *FABP4* = fatty acid binding protein 4; *TGFB3* = transforming growth factor 3; *BMP2* = bone morphogenic protein 2.

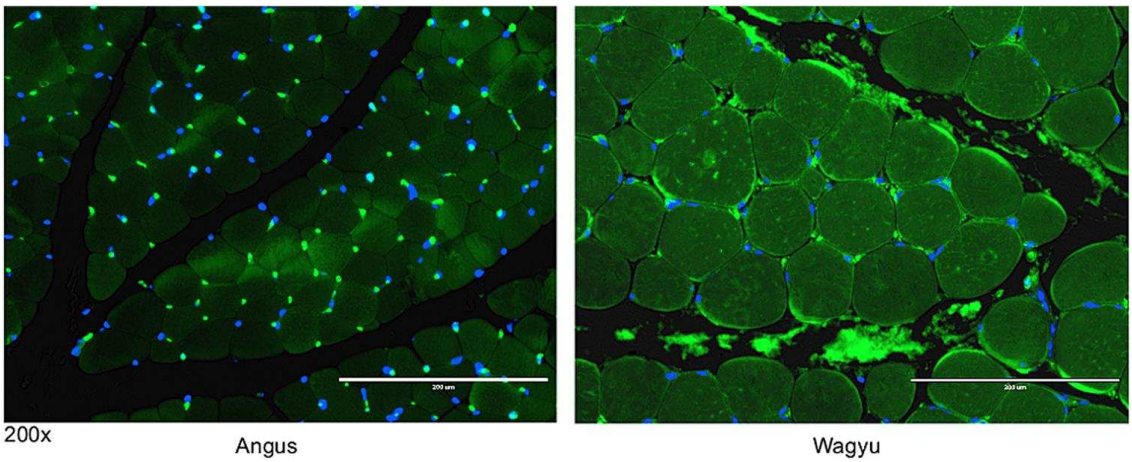

Figure 1. Immunofluorescence staining showed a greater number of fatty acid binding protein (FABP4) positive cells between muscle fibers and muscle bundles in Wagyu when compared with Angus skeletal muscle (Sternomandibularis muscle): FABP4 stained green and nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI). (Adapted from figure 5 in reference Duarte et al., 2013, with permission. Figure provided by M. S. Duarte).

Fat deposition in cattle typically follows the order in which perirenal fat is deposited first, followed by intermuscular, subcutaneous and finally by the IMF (Sainz and Hasting, 2000; Pethick et al., 2005 and 2007; Hocquette et al., 2010). Thus, as IMF deposition is time dependent in most cattle breeds and is dependent on a high energy intake, the use of cattle breeds, instead of Wagyu as models for human dysfunctions is compromised, since it would not be possible to dissociate the effect of energy intake or age from the genetic predisposition for IMF deposition abnormalities. Therefore, the use of animals that have a unique ability to deposit IMF regardless of energy intake or age, such as Wagyu cattle, may be useful in studies of adipogenesis (Lehnert et al., 2006). In summary, Wagyu cattle have a great potential to be used as animal models for adipogenesis studies due to their exceptional IMF deposition (Shirouchi et al., 2014), which in turn affects carcass composition. Comparison of Wagyu animals with other popular beef breeds, such as Angus, provides a distinct basis for observation, as Wagyu

presented higher feed efficiency, feed-to-gain ratio, and overall greater IMF deposits (Shirouchi et al., 2014). This can be seen in Figure 1, which shows an immunofluorescent staining comparison of the adipogenic marker FABP4 in Angus and Wagyu in the Sternomandibularis muscle, where the number of preadipocytes and adipocytes in Wagyu was greater than that of Angus cattle. Laboratory identification of distinct differences between Wagyu cattle and any comparable beef breed produces valuable data, which can further identify factors that either increase or inhibit adipogenesis and IMF deposition. Research with tissue and cells from Wagyu animals, can be a model to deep understanding of adipogenesis and lipid metabolism, which might be paralleled by similar studies in tissues and cells from other animal types.

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CHAPTER III

GENE EXPRESSION IN ADIPOGENIC PROGENITOR CELLS FROM DIVERGENT PIG BREEDS

(Article to be submitted)

ABSTRACT

Excessive reduction of fat in pork throughout the years has implied in qualitative reduction of the final product and consequently disadvantage to the industry due to the low acceptance by the consumer market. Fat deposition occurs through adipogenesis, the process when progenitor cells are recruited to adipogenic lineage, considered a term describing proliferation, differentiation, and conversion of cells into lipid assimilating cells. Piau breed animals represent an excellent biological model for studies related to adipogenesis by presenting high fat content, these animals have high potential for use as a source of fat cells to be used on *in vitro* studies. Research on primary cell cultures would provide a better understanding of the mechanisms responsible for characterizing Piau breed as a source of genetic diversity in contrast to other breeds used in breeding programs due to its increase in adiposity of the carcass and meat. The present study was conducted to evaluate the gene expression differences between a commercial pig line and Piau breed, regarding adipogenic genes (*ADIPOQ*, *LPL*, *SCD1*, *FASN* and *ACACA*) and transcription factors (*CEBPA, PPARG* and *SREBP1*) involved in differentiation of preadipocytes into mature adipocytes.

Keywords: adipogenic lineage**,** lipid, pork, preadipocytes

RESUMO

A redução excessiva de gordura na carne suína ao longo dos anos tem implicado na redução qualitativa do produto final e, consequentemente, prejudicando a indústria devido à baixa aceitação pelo mercado consumidor. A deposição de gordura ocorre através da adipogênese, que é o processo pelo qual as células progenitoras são recrutadas para a linhagem adipogênica, e considerado um termo que descreve a proliferação, diferenciação, e a conversão destas em células acumuladoras de lipídeos. Animais da raça Piau representam um excelente modelo biológico para estudos relacionados à adipogênese, por apresentarem um elevado teor de gordura, estes animais possum um elevado potencial para serem utilizados como uma fonte de células de gordura a serem utilizadas em estudos *in vitro*. A obtenção de resultados a partir de culturas primárias de células permite uma melhor compreensão dos mecanismos responsáveis pela caracterização da raça como uma fonte de diversidade genética em contraste com outras raças utilizadas em programas de melhoramento devido ao seu aumento de adiposidade da carcaça e da carne. O presente estudo foi realizado para econtrar diferenças de expressão gênica entre uma linhagem commercial de suíno e a raça Piau, em relação genes adipogênicos (*ADIPOQ, LPL, SCD1, FASN* and *ACACA*) e fatores de transcrição (*CEBPA*, *PPARG* and *SREBP1*), envolvidos na diferenciação dos pré-adipócitos em adipócitos maduros.

Palavras-chave: carne suína, linhagem adipogênica, lipídeos, pré-adipócitos

INTRODUCTION

Substancial advances in the pork industry have been achieved over the years through genetic selection of animals with high yield and lean gain. However, the intensity of animal selection programs to improve feed efficiency and animal performance is indicated as one of the main factors responsible for deterioration of the sensory aspect of pork, which is seen as a problem for industry in countries like United States and Canada (Dodson et al., 2010). This is partly because of the fat, especially intramuscular, that is responsible for providing juiciness, taste, flavor and tenderness to the meat (Duarte et al., 2013). Thus, excessive reduction of fat in pork implies qualitative reduction of the final product and consequently affecting negatively industry due to the low acceptance thereof by the consumer market. Fat deposition occurs through adipogenesis, the process when progenitor cells are recruited to adipogenic lineage, considered a term describing proliferation, differentiation, and conversion of cells into lipid assimilating cells found within fat tissue (Hausman et al., 2009).

Local breeds, as Piau, have been used as models to understand fat deposition in the pig (Peixoto et al., 2006). Piau local breed has low commercial value presenting genetic characteristics favorable to the qualitative aspect of the meat such as IMF (Serão et al., 2011). A number of studies from our research group have provided substantial comparative information between Piau and commercial pig lines, with different objectives as QTL mapping using different crossbreds with Piau breed (Pita et al., 2003; Pires et al., 2006; Pires et al., 2007; Paixão et al., 2008; Silva et al., 2008; Silva et al., 2009; Paixão et al., 2012), analysis of polymorfisms of candidate genes (De Oliveira Peixoto et al., 2006; Soares et al., 2006; Figueiredo, 2008; Faria et al., 2009),

performance and carcass traits (De Oliveira Band et al.; De Oliveira Peixoto et al., 2006; Faria et al., 2009), phylogeny and genetic diversity (Schierholt et al., 2008; Sollero et al., 2009; Souza et al., 2009), muscle development (Sollero et al., 2011; Verardo et al., 2012) and expression patterns of candidate genes (Serão et al., 2011; Nascimento et al., 2012). However, despite the considerable knowledge generated to date about this breed and its applications, there is a lack of knowledge of cellular mechanisms that confer the Piau breed high fat deposition.

Thus, since Piau breed animals represent excellent biological model for studies related to adipogenesis (Verardo et al., 2012) by presenting high fat content (Serão et al., 2011), these have high potential for use as a source of fat cells to be used on *in vitro* studies. Research on primary cultures would provide a better understanding of the mechanisms responsible for characterizing the breed as a source of genetic diversity in contrast to white breeds used in breeding programs due to its increase in adiposity of the carcass and meat. The aim of this study was to reveal the gene expression differences between the commercial pig line and Piau breed, regarding adipogenic genes involved in differentiation of preadipocytes into mature adipocytes.

MATERIALS AND METHODS

All animal procedures were performed according to protocols approved by the Committee for Institutional Use of Animals in Research from Universidade Federal de Viçosa (Viçosa, MG – Brazil), process number 17/2013.

Animals and sampling

The animals used in this study were female pigs from a commercial pig line (n=3) and Piau breed (n=3), maintained at Granja de Melhoramento de Suínos of Department of Animal Science in Universidade Federal de Viçosa. Subcutaneous fat samples were collected from animals slaughtered, kept in warm PBS and transported to Animal Biotechnology Laboratory (LABTEC) to be processed.

Isolation of stromal vascular cells

Cells from the stromal fraction of the backfat were collected from the animals and isolated to obtain stromal vascular cell cultures. Briefly, fat tissue was minced and digested in 0.25% collagenase solution (Collagenase Type I – Gibco™, Whaltham, USA) at 37ºC for 1h with gentle agitation. The digested cell suspension was then filtered through 1000 μm nylon meshes, allowing the free cells to pass and retaining the undigested tissue. Cells were separated by centrifugation, and the stromal vascular cells stay at the bottom of the conical tubes, according to Fernyhough et al. (2004). Washed cells were then transferred to a 25 cm^3 cell culture bottles (Sarstedt™ - Newton, USA) fulfilled with Dulbecco's modified Eagle's medium nutrient mixture (DMEM/F12 - Gibco™, Whaltham, USA) in a 1:1 ratio, supplemented with 10% Fetal Bovine Serum (FBS - Gibco™, Whaltham, USA) and antibiotics (1% Pen/Strep; 0.5% Gentamicin - Gibco™, Whaltham, USA). Cells were cultured under incubation in humidified 5% CO_2 atmosphere and the medium was changed every two days until the confluence was reached. After that, cells were harvested (Tryple selection 10x – Gibco™, Whaltham, USA), resuspended with cryopreservation medium (DMEM supplemented with 20%

Horse Serum and 10% DMSO – Sigma-Aldrich™- St. Louis, USA) and cryopreserved in liquid nitrogen until induction protocols experiments.

Adipogenic induction

Cells from the six animals were thawed and cultured in 25cm^3 volume cell culture bottles (n=4 per animal), in a number of 4x10^4 cells. Culture medium (DMEM/F12 plus antibiotics) was changed every 2 days until they reached 50-60% confluence. Then we applied an adipogenic induction cocktail, composed by the regular medium added by IBMX (0.25mM), dexamethasone (0.5µM) and insulin (1µg/µL), so half of the bottles were submitted to induction of adipogenic protocol and half of the bottles were submitted to spontaneous differentiation. We waited for 10 days to collect the cells.

Lipid accumulation investigation

We performed Oil Red-O staining at 10 days after beginning the induction protocol to investigate intracellular lipid accumulation, to evidence intracytoplasmatic lipid content (Ramirez-Zacarias et al., 1992). Briefly, cells were washed three times with phosphate buffered saline (PBS) followed by fixation with 10 % formalin in phosphate buffer for 1 h at room temperature. Then, fixed cells were washed once with PBS and stained with a filtered Oil Red-O (Sigma-Aldrich™, St. Louis, USA) working solution prepared from dilution of stock solution (0.5g of Oil Red-O in 100mL of isopropyl alcohol) for 15 min at room temperature. Plates were then washed twice with distilled water prior to microscopy observation.

RNA extraction and cDNA synthesis

In the tenth day after adipogenic induction, cells were collected from the bottles using 1mL of TRIZOL™ reagent (Life Technologies, Eugene, USA) per bottle. Total RNA was extracted according to manufacturer's recommendations and then quantified in a spectrophotometer NanoVue Plus™ (GE Healthcare, Little Chalfont-UK) observing A260/A280 ratios between 1.8 and 2.0 as purity control. Quality and integrity of extracted RNA were verified using 1% agarose gel stained with ethidium bromide. Total RNA was reverse transcribed immediately into cDNA with Go ScriptTM Reverse Transcription System Kit (Promega, Fitchburg-USA), according to the manufacturer's recommendations.

Gene network and Reverse Transcription Quantitative PCR (RT-qPCR) Analysis

Based on literature review (Ducharme and Bickel, 2008; Hausman et al., 2009; Samulin et al., 2009; Gondret et al., 2011; Ladeira et al., 2016), a set of transcription factors (TF) and genes were selected for the expression analysis: Peroxisome Proliferator- activated Receptor gamma (*PPARG*), CAAT/Enhancer Binding Protein alpha (*CEBPA*), Sterol Regulatory Element Binding Transcription Factor 1 (*SREBP1*), Adiponectin (*ADIPOQ*), Diacylglycerol O-Acyltransferase 2 (DGAT2), Carnitine Palmitoyltransferase 1A (*CPT1A*), Lipoprotein Lipase (*LPL*), Stearoyl-CoA Desaturase 1 (*SCD1*), Adipose Triglyceride Lipase (*ATGL*), Fatty Acid Synthase (*FASN*), Acetyl-CoA Carboxylase alpha (*ACACA*) and Solute Carrier Family 2 (Facilitated Glucose Transporter) member 4 (*SLC2A4*).

ClueGO Cytoscape plug-in (Bindea et al., 2009) was used, based on one-sided hypergeometric test and Bonferroni correction, to construct a gene network highlighting the Pathways and relations across both TF and genes (Figure 1). According to main networks, we observed two TF (*PPARG* and *CEBPA*) and one gene (*ADIPOQ*) sharing differentiation of white and brown adipocytes. We also observed two TF (*PPARG* and *SREBP1*) and four genes (*LPL*, *SCD1*, *FASN* and *ACACA*) sharing the SREBP signaling pathway. From these genes, three (*SCD1*, *FASN* and *ACACA*) are also involved with fatty acid biosynthesis. Thus, based on these main pathways networks we selected three TF (*PPARG*, *CEBPA* and *SREBP1*) and five genes (*ADIPOQ*, *LPL*, *SCD1*, *FASN* and *ACACA*) to perform qRT-PCR, using β-actin (*BACT*) as a housekeeping gene.

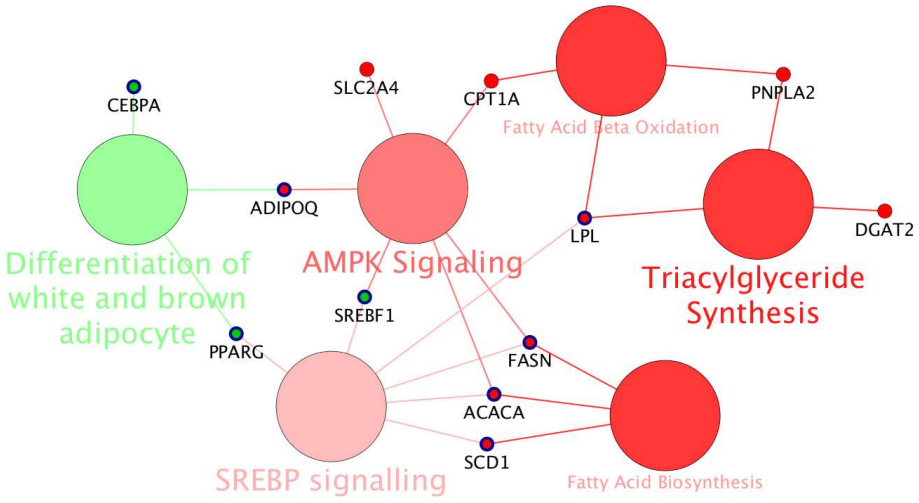


Figure 1. Main functional group networks with pathways terms of genes and transcription factors (TF) as nodes. Green nodes represent Pathways of transcription factors while red nodes are related to genes. Pink light nodes are Pathways shared between both genes and TF. The most enriched terms per group are shown in bold according to enrichment significance from ClueGO Cytoscape plug-in. Nodes with blue border are genes and TF used for qRT-PCR.

RT-qPCR reactions were performed in ABI Prism 7300 Sequence Detection Systems Thermocycler™ (Applied Biosystems, USA), using GoTaq™ qPCR Master Mix Kit (Promega™), according to manufacturer's protocol. The RT-qPCR condition was as follows: 95°C for 2 min; 40 denaturation cycles at 95°C for 15 s, annealing and extension at 60°C for 60 s. Amplification efficiency of primers were tested, adding an additional step (raising temperature from 60° to 94°C), in order to obtain a dissociation curve. Experiment was conducted with two biological and three technical replicates, for each treatment in a single 96-well plate. Relative mRNA level was analyzed with $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with *BACT* as the endogenous control. Primers were designed to span genomic regions using PrimerQuest (http://www.idtdna.com/primerquest/Home/Index) with sequences described in Table 1.

Table 1. List of primers used to RT-qPCR of genes involved in adipogenesis

Gene	Primer Sequence (5'-3')	Acession Number NCBI
ACACA	F: ATGAAGGAGGAGGTGGATAG R: ACCCTTAGGACCAGTAAGAC	EU168399.1
ADIPOQ	F: TGATGGCAGAGATGGCGTCC R: ACCCTTAGGACCAGTAAGAC	AY589691.1
CEBPA	F: ATCAGGCAGAGAGGAGAAG R: CCAAGGCACAGGGTTATTC	XM_003127015.2
FASN	F: TGAAGAGGTCCTTCTACGG R: ACCCAACGGAAGCTAGT	EF589048.1
LPL	F: TGGAGTGACAGAATCTGTGG R: CATTCCTGTCACCGTCCAGCC	NM_214286.1
PPARG	F: CTGAGAAGGAGAAGCTGTTG R: CAGCGGGAAGGACTTTATG	AB097930.1

SCD1	F: CAAAGAGAAGGGTGGTTTGC R: GGTTTGTAGTACCTCCTCTGG	NM_213781.1
SREBP1	F: GCTCCTCCATCAATGACAAG R: GCAAGACGGCGGATTTAT	AY496867.1
BACT	F: CTTCTAGGCGGACTGTTAGTTG R: AGCCATGCCAATCTCATCTC	AY550069.1

Statistical Analysis

Gene expression data from the housekeeping gene and target genes were generated in Ct (Cycle threshold) values. Then, these data were analyzed using the macro %QPCR_MIXED developed in SAS (www.msu.edu/~steibelj/JP_files/QPCR.html) according to the metodology proposed by Steibel et al. (2009), where the Ct individual values for each target gene were normalized based on Ct values of housekeeping gene. The results were submmited to variance analysis according to the following model:

$$Y_{gijk} = G_{ig} + I_{jg} + (G \times I)_{gij} + B_{ijk} + e_{gijk}$$

Where:

Y_{gijk} = measured expression level of the g^{th} gene (Ct);

G_{ig} = effect of i^{th} genetic group in the g^{th} gene;

I_{jg} = effect of j^{th} protocol of adipogenic induction in the g^{th} gene;

$(G \times I)_{gij}$ = effect of interaction between the i^{th} level of G and the j^{th} level of I in the g^{th} gene;

B_{ijk} = random sample-specific effect of the k^{th} cell culture bottle common to both genes (target and housekeeping);

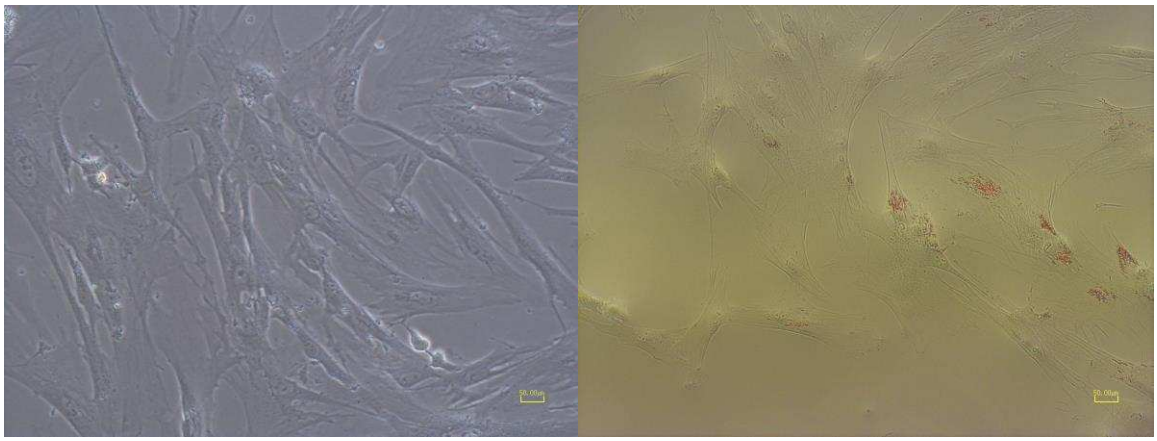
e_{gijk} = random effect.

To test differences and interactions between factors in the expression rate of genes of interest normalized by the endogenous gene (*BACT*), different contrasts were performed between the genetic groups and induction protocols (ΔΔCT). Significance of ΔΔCT estimates were determined by the Student's t-test at level of 5% of probability. Finally, the differences in fold change (FC) were estimated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

RESULTS

Isolation of stromal vascular cells and lipid accumulation investigation

Cells were successfully isolated, frozen and kept in liquid nitrogen until the following experiments. In order to check deposition of lipids, the cells were stained with the Oil Red-O staining, and this accumulation was confirmed by the visualization of red small dots inside the culture, as seen in Figure 2. All bottles presented intracellular lipids, from induction adipogenic protocol and from spontaneous protocol, but the last ones in a small proportion.



(a) (b)

Figure 2. Stromal vascular cells in culture after 10 days of induction with an adipogenic cocktail; (a) cells before Oil Red-O staining, (b) cells after Oil Red-O staining.

RNA extraction, cDNA synthesis and RT-qPCR Analysis

Samples of RNA were quantity and quality checked (data not shown), after that all samples were considered useful for the downstream analysis. Interaction between genetic groups and induction protocols was observed for gene *ADIPOQ* (P=0.02). We also observed difference between genetic groups (P=0.02), when cells were submitted to induction of adipogenic protocol what showed a higher expression for cells from animals of commercial pig line (FC=5.39). Consequently, the expression was lower in Piau breed. No difference between genetic groups (P=0.26) was found when cells were submitted to spontaneous protocol. Regarding to commercial line cells, the higher expression of this gene was found in cells submitted to induction of adipogenic protocol (FC=4.5).

For *SCD1* gene we observed interaction between genetic groups and induction protocols (P=0.03). Considering cells submitted to induction protocol, cells of

commercial pig line showed higher *SCD1* gene expression than cells from Piau breed (P=0.05, and FC=2.01). In addition, we did not observe difference (P=0.29) in cells submitted to non-induction protocol. We did not find differences when comparing protocols of induction neither in commercial line nor in Piau breed (P=0.0612 and P= 0.3367, respectively).

Regarding gene expression levels of *PPARG* transcription factor, there was no interaction (P=0.60) between genetic groups and induction protocols. We neither observed difference between genetic groups (P=0.28), nor between induction protocols (P=0.42). Similarly, for genes *ACACA*, and *FASN* we did not find interaction between genetic groups and induction protocols (P=0,09, P=0.60, respectively), neither between genetic groups (P=0.11 and P=0.26, respectively), nor between induction protocols (P=0.12, P=0.88).

For *CEBPA* transcription factor, there was no interaction between genetic groups and induction protocols (P=0.15). In contrast, we observed a significant difference between genetic groups (P=0.02), being higher expression in commercial pig line cells comparing to cells from Piau origin (FC=2.99), and between the induction protocols, no difference was observed (P=0.75).

The *LPL* gene did not show interaction between genetic groups and induction protocols (P=0.06). No difference was found between genetic groups (P=0.13). However, we observed a significant difference between induction protocols (P=0.04), being the higher expression observed in the cells that were submitted to induction of adipogenic protocol (FC=2.30).

For *SREBP1* there was no interaction (P=0.18) between genetic groups and induction protocols. Regarding to genetic groups we found a difference (P=0.04), being the lower expression presented for cells from commercial pig line (FC=2.94). No difference was found between the induction protocols (P=0.96).

DISCUSSION

Mesenchymal stem cells (MSCs) become preadipocytes when they lose their ability to differentiate into other mesenchymal lineages and become committed to the adipocytic lineage. This initial phase of adipocyte differentiation is known as determination and is poorly characterized. The second phase of adipogenesis is terminal differentiation, whereby preadipocytes take on the characteristics of mature adipocytes, acquiring lipid droplets and the ability to respond to hormones such as insulin (Christodoulides et al., 2008).

Regarding to lipid accumulation, since the cells were stained by Oil Red-O staining, the results show us that the cells differentiated and started to accumulate fatty acids after 10 days of culture, in accordance with Huang et al. (2012) that reported the use of the same staining technique in bovine stromal vascular cells where they visualized adipogenic differentiation after 12 days of induction by the presence of adipocytes. Qu et al. (2015) showed cells with visible signs of lipid accumulation from day 6 of differentiation and were fully lipid filled by day 9, also using Oil Red-O staining.

Terminal differentiation of preadipocytes consists of a cascade of transcriptional events (Rosen and MacDougald, 2006). According to the results found in this study, the

genes *ADIPOQ*, *SCD1*, *CEBPA*, *LPL* and *SREBP1* showed significant differences between the genetic groups or induction protocols.

A previous notion of adipose tissue as little more than storage depots for body energy was recently challenged with the discovery of adiponectin (*ADIPOQ)* and leptin (Haluzík et al., 2004). These discoveries firmly established adipose tissue as an endocrine organ and concurrently propelled adipogenesis to the forefront of scientific research (Linehan et al., 2012). *ADIPOQ* is an important adipokine involved in the control of fat metabolism and insulin sensitivity, which stimulates AMPK phosphorylation and activation in liver and skeletal muscle, enhancing glucose utilization and fatty-acid combustion. It is also an important *in vitro* signaling molecule that regulates adipocyte differentiation in adipose tissue (Gao et al., 2013).

In this study we observed higher expression of *ADIPOQ* in cells from commercial pig line in comparison to cells from Piau breed, and also between induction protocols. When *ADIPOQ* gene is silenced, according to Gao et al. (2013), the number of lipid droplets in the adipocytes was significantly reduced in transfected group, whereas cells of the negative control and untransfected groups showed increased numbers of lipid droplets and larger droplet sizes. Since A*DIPOQ* is a gene expressed in adipose tissue exclusively, we can highlight that differentiation occurred and the preadipocytes became adipocytes and started to deposit lipids faster when we used the induction to adipogenic protocol and in cells from commercial pig line.

Many studies have reported similar results as Gao et al. (2013) who reported that decreasing the expression of *ADIPOQ* suppresses the differentiation of preadipocytes and

reduces lipid accumulation. Furthermore, Fu et al. (2005) demonstrated that overexpression of *ADIPOQ* can enhance 3T3-L1 fibroblast proliferation, accelerate adipocyte differentiation, and in fully differentiated adipocytes, augment lipid accumulation and Combs et al. (2001) demonstrated that the overexpression of *ADIPOQ* can enhance the proliferation, differentiation and lipid accumulation of 3T3-L1 cells, so *ADIPOQ* functions as an adipocyte differentiation factor.

Our current findings support a difference between cells from commercial pig line and Piau breed and taking into account that the background of the donors can influence the performance of cultured cells, the observed higher expression of *ADIPOQ* by cells from commercial pig line animals when using an induction of adipogenic protocol, might respect a correlated lipid accumulation in adipocytes from this line.

Gene expression studies in humans and animals have shown that elevated stearoyl-CoA desaturase (*SCD1*) activity is associated with increased fat accumulation and monounsaturation of saturated fatty acids in skeletal muscle (Jiang et al., 2008). *SCD1* is one of the key lipogenic enzymes, responsible for desaturation of saturated fatty acyl-CoAs, the preferred substrates being palmitoyl- and stearoyl-CoA, which are converted to palmitoleoyl- and oleoyl-CoA, respectively. These monounsaturated fatty acids are used as substrates for the synthesis of triglycerides, wax esters, cholesteryl esters and membrane phospholipids (Miyazaki and Ntambi, 2003). Our results are in accordance with Wang et al. (2004) that reported *SCD1* gene as highly expressed in adipocytes compared with preadipocytes, which we can see by comparison of induction of adipogenic and spontaneous protocol. Similarly, Ralston et al. (2014) provided new mechanistic insight into the role of *SCD1* as a regulator of fatty acid profiles and

triacylglycerides synthesis in adipocytes, and concluded that inhibition of the enzyme downregulated genes affecting triacylglycerol synthesis. These findings also corroborate that in our study, differentiation occurred supported by overexpression of *SCD1* gene in cells from commercial pig line compared to cells from Piau breed, when submitted to induction of adipogenic protocol.

CEBPA is known as one master transcriptional regulator of adipogenic cascade (Gondret et al., 2011), acting as a key regulator of adipocyte differentiation (Rosen et al., 2002) and it is highly expressed in many tissues (Lekstrom-Himes and Xanthopoulos, 1998) serving as a transcriptional activator in adipocyte differentiation (Darlington et al., 1998). Our results showed that *CEBPA* was overexpressed in cells from commercial pig line in relation to cells from Piau breed, indicating increased adipogenic differentiation.

CEBPA expression is influenced by *PPARG* expression, which promotes the differentiated phenotype, at least in part by increasing the expression of *PPARG* in a classic example of positive feedback (Rosen et al., 1999). Despite the high degree of cooperativity between *PPARG* and *CEBPA* in adipocytes, some adipocyte genes are clearly more dependent on *CEBPA* than on *PPARG*, and vice versa (Lefterova et al., 2014), this can explain why in this study we did not find differences in *PPARG* gene, differently for *CEBPA*. McNeel and Mersmann, (2000) showed that *CEBPA* regulates the expression of adipocyte-characteristic genes such as lipoprotein lipase (*LPL*), leptin, adipocyte fatty acid binding protein (*FABP4*), and fatty acid synthase (*FASN*).

LPL is an adipogenic enzyme, expressed at high levels in adipose tissue and hydrolyzes triglyceride-rich lipoproteins at the capillary endothelium to generate non-

esterified fatty acids for uptake in peripheral tissues (Linehan et al., 2012). In our study we showed that the expression of *LPL* was significantly different between induction protocols. The higher expression of *LPL* gene in cells that were submitted to the induction of adipogenesis reflects an increase in lipid storage by these cells, which is in accordance with Linehan et al. (2012) that studied the necessity of *LPL* expression by adipocytes for intracellular lipid accumulation in vitro.

According to Taniguchi et al. (2008), *SREBP1* gene plays an important role in preadipocyte differentiation. Similarly, Arasu et al. (2014) reported that S*REBP1* gene is mainly involved in promoting proliferation and differentiation of adipocytes, regulating fatty acid metabolism and stimulating genes that participate in the lipogenesis by fatty acid synthase, acetyl carboxylase, glycerol-3-phosphate acyltransferase, and the lipoprotein lipase. In our study, *SREBP1* was the only gene overexpressed in cells from Piau breed compared to cells from commercial pig line. Similarly, Zhang et al. (2014) reported that *SREBP-1c* mRNA expression was higher in Bamei, possess a strong ability to deposit fat, than in Landrace breed in both subcutaneous and intramuscular adipocytes.

Considering the difference of cell origin influencing the expression of several genes on *in vitro* studies and consequently distinct behavior of cells, such as the findings of the present study for adipogenic genes, a significant difference is also expected *in vivo*, as reported by Verardo et al. (2012), who found differentially expressed genes in skeletal muscle development comparing Duroc, Large White and Piau breed. Similarly, Sollero et al. (2011) studied fetal pig skeletal muscle revealing developmental patterns of gene expression, being a large number of differentially expressed genes both between

developmental ages and between breed types. In general, the developmental transcript profiles for Piau breed and Yorshire-Landrace pigs were similar, although breed-specific patterns of gene expression were revealed.

Such differences in gene expression could be influenced by post-transcriptional factors, such as micro-RNAs (miRNAs), that have been implicated in partial or full silencing of respective target genes in many different species and gene pathways (Zeng, 2006; Arias et al., 2015). Regarding adipogenesis, many studies have reported the importance of miRNAs in regulatory control over gene expression, such as Peng et al. (2014) who reported several miRNAs influencing adipogenesis and lipid metabolism. In our study, *ADIPOQ*, *SCD1*, *CEBPA*, *LPL* and *SREBP1* genes presented significant differences between genetic groups or induction protocols, which may be explained by these post-transcriptional mechanisms, demanding future studies.

Commercial pig line cells presented higher expression of adipogenic genes after ten days of adipogenic protocol. These results can be explained by the fact that these cells are more sensitive to the cocktail than Piau breed cells, which are acquainted with lipid accumulation, so the responses were not so evident. Summarizing, genes involved in lipid metabolism can be expressed or not depending on the origin, interaction, confluence and collecting time of cells, besides specific marks and post-transcriptional factors as miRNAs can be also enrolled. Our study cooperates to the understanding of some molecular mechanisms involved in adipogenesis.

CONCLUSIONS

Since commercial pig line has been selected through many years for higher deposition of lean meat, the use of an adipogenic cocktail can stimulate cells to be commited to adipogenic lineage than cells that already come from high fat deposition breeds. Our study suggests that when cells are isolated and cultured *in vitro* they can present atypical patterns of expression depending on their origin, showing different response. The study also indicates that cells from Piau breed behave differentially from commercial animals cells when comparing *in vitro* studies, presenting lower expression of genes related to adipogenic lineage cultured in a chemical induction environment. Further *in vitro* studies are needed to confirm differential expression between these two genetic divergent groups.

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To CAPES, CNPq, FAPEMIG.

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CHAPTER IV

VITAMIN A EFFECTS ON GENE EXPRESSION AND PROTEOMIC PROFILES IN CATTLE

(Article to be submitted)

ABSTRACT

Vitamin A is a key regulator of gene expression, influencing adipogenesis and lipid metabolism in animal tissues. Although the mechanisms by which retinoic acid (RA), a metabolite of vitamin A, regulates energy homeostasis and lipid metabolism in mature adipocytes are well understood, little information is available on its functions in preadipose cells, at the early commitment. Enhancing adipogenesis of progenitor cells increases intramuscular fat (marbling), consequently improving palatability of meat. The quality and value of the carcass in domestic meat animals are reflected in its protein and fat content. Proteomics studies based two dimensional electrophoresis have been performed to investigate the proteins involved in muscle to meat conversion, and it is a method of choice for the quantitative differential display of large numbers of proteins, being a promising and powerful tool in meat science. In the present study, we performed a gene expression experiment in stromal vascular cells from adipose tissue to elucidate the effect of RA on the early commitment in those cells to adipogenic lineage, using *CEBPA, CEBPB, Zfp423* and *PPARG* transcription factors, and *FABP4* and *TGFB* genes, revealing a decreased expression of *Zfp423* and *FABP4*. Secondly, as a validation experiment, we conducted a proteomic essay looking for effects of vitamin A supplementation *in vivo*, showing that vitamin A enhances the abundance of structural muscle proteins. Regarding intramuscular fat (IMF), it can be concluded that the present study cooperates elucidate gene expression in adipogenic differentiation, and also indicates that to increase IMF, dietary vitamin A levels have to be controlled.

Keywords: marbling, retinoic acid, proteomics

RESUMO

A vitamina A é um regulador chave da expressão gênica, influenciando a adipogênese e o metabolismo de lipídeos nos tecidos animais. Embora os mecanismos pelos quais o ácido retinóico (RA), um metabólito da vitamina A, regula a homeostase energética e o metabolismo lipídico em adipócitos maduros são bem compreendidos, mas há pouca informação disponível sobre as suas funções em células pré-adiposas, no comprometimento inicial. Melhorar a adipogênese de células progenitoras aumenta a gordura intramuscular (marmoreio), consequentemente, melhorando a palatabilidade da carne. A qualidade e o valor da carcaça dos animais no mercado interno são refletidas no seu conteúdo de proteína e gordura. Estudos de proteômica baseados em 2DE (eletroforese bidimensional) tem sido realizados para investigar as proteínas envolvidas na conversão de músculo em carne, e é um método de escolha para a apresentação diferencial quantitativa de um grande número de proteínas, além de ser uma ferramenta promissora e poderosa na ciência da carne. No presente estudo, foi realizado um estudo de expressão gênica em células da fração vascular estromal do tecido adiposo para elucidar os efeitos do RA no comprometimento inicial destas células à linhagem adipogênica utilizando fatores de trascrição *CEBPA*, *CEBPB*, *Zfp423* e *PPARG*, e os genes *FABP4* e *TGFB*. Observou-se redução na expressão de *Zfp423* e *FABP4*, e posteriormente, como um experimento de validação, realizamos um ensaio proteômico procurando efeitos da vitamina A na suplementação *in vivo*, mostrando que a vitamina A aumenta a abundância de proteínas estruturais. Em relação à gordura intramuscular (IMF), conclui-se que o presente estudo colabora com o esclarecimento da expressão gênica na diferenciação adipogênica, e também indica que para aumentar a IMF, os níveis de vitamina A na dieta devem ser controlados.

Palavras-chave: ácido retinóico, marmoreio, proteômica

INTRODUCTION

Marbling, or intramuscular fat, has been consistently identified as one of the top beef quality problems (Du et al., 2010). Intramuscular adipocytes are distributed throughout the perimysial connective tissue of skeletal muscle and are the major sites for the deposition of intramuscular fat, which is essential for the eating quality of meat. Stromal vascular cell cultures combined with molecular biology investigations have provided insights into mechanisms regulating fat deposition (Hausman et al., 2009). It has been well established that stromal vascular cells are major sources of adipogenic cells in skeletal muscle. Enhancing adipogenesis of progenitor cells increases intramuscular fat (marbling), improving palatability of meat (Du et al., 2013).

Adipogenesis is regulated by a cascade of events, influenced by expression of particular adipogenic genes and transcription factors (Huang et al., 2012), such as CCAAT/enhancer binding proteins (CEBPs), fatty acid binding protein 4 (*FABP4* or *Ap-2* gene), Zinc finger protein 423 (*Zfp423)* and Peroxisome proliferator activated receptor gamma (*PPARG).* Genes related to adipogenesis should be deeply studied due to the fact that differentiation of adipocytes is a complex event with many factors and signaling pathways involved and the exact mechanisms that induce adipogenesis *in vivo* remain not fully elucidated (Gustafson et al., 2015). Similarly, the effect of different diet components that might influence the deposition of fat, such as amino acids and vitamins, is not well understood.

Vitamin A is a key regulator of gene expression, influencing adipogenesis and lipid metabolism in animal tissues. Retinoic acid (RA) is derived from vitamin A, and it

is a ligand for both retinoic acid receptor (RAR) and *PPARG* (Lefterova and Lazar, 2009). Although the mechanisms by which RA regulates energy homeostasis and lipid metabolism in mature adipocytes are well understood, little information is available on its functions in preadipose cells at early commitment.

Since quality and value of carcass in livestock are reflected in its protein and fat content (Hausman et al., 2009) proteomics studies based 2DE (two dimensional electrophoresis) has been performed to investigate the proteins involved in muscle to meat conversion. It is also a method of choice for the quantitative differential display of large numbers of proteins besides being a promising and powerful tool in meat science (Murgiano et al., 2010).

In the present study, we performed an *in vitro* gene expression experiment on cattle stromal vascular cells to elucidate the effect of retinoic acid on the primary commitment to the adipogenic lineage. Secondly, as a validation *in vivo* experiment, we conducted a proteomic assay in order to evaluate the effect of retinoic acid on proteomic profiles in the *Longissimus dorsi* of animals supplemented or not with vitamin A.

MATERIALS AND METHODS

All animal procedures were performed according to protocols approved by the Committee for Institutional Use of Animals in Research from Universidade Federal de Viçosa (Viçosa, MG – Brazil).

1. Cell culture, RNA extraction and cDNA synthesis

In order to test the effect of RA *in vitro*, bovine stromal vascular (SV) cells were obtained from the stromal vascular fraction of adipose tissue (a source of different kinds of cells) from Angus breed animals and cultured at low density. After 20% confluence, cells were treated with the demethylation agent 5-Aza-2′-deoxycytidine (Sigma-Aldrich, St.Louis, Missouri-USA) allowing the dedifferentiation, as newly pluripotent, to be committed to different types of cells. After 80-100%, confluence cells were treated with ATRA (all-trans retinoic acid) (Sigma-Aldrich, St.Louis, Missouri-USA) . After 48 hours, cells were collected for extraction of RNA. Total RNA was extracted using 1 mL of TRIZOL™ reagent protocol (Life Technologies, Eugene, USA) according to the manufacturer's instructions. RNA samples were quantified in a spectrophotometer NanoVue Plus™ (GE Healthcare, Little Chalfont, UK) observing A260/A280 ratios between 1.8 and 2.0 as purity control. Quality and integrity of extracted RNA were verified using 1% agarose gel stained with ethidium bromide. Total RNA was reverse transcribed immediately into cDNA with Go ScriptTM Reverse Transcription System Kit (Promega, Fitchburg-USA), according to the manufacturer's recommendations.

2. Gene expression and Western Blotting Analysis

We used qRT-PCR to analyze the following genes: Peroxisome Proliferator-activated Receptor gamma (*PPARG*), CAAT/Enhancer Binding Protein alpha (*CEBPA*), CAAT/Enhancer Binding Protein beta (*CEBPB*), Zinc-finger protein 423 (*Zfp423*), Fatty Acid Binding Protein 4 (*FABP4*) and Transforming Growth Factor beta (*TGFB*), using

18S ribosomal RNA (*18S*) as the housekeeping gene. Primers were designed using PrimerQuest (http://www.idtdna.com/primerquest/Home/Index) and are listed in Table 1.

Table 1. List of primers used for qRT-PCR analysis

Gene	Primer Sequence (5'-3')	NCBI Acession Number
CEBPA	F: ATGAGCAGCCACCTCCAGA R: GCCAGGAACTCGTCGTTGAA	NM_176784.2
CEBPB	F: CCAACTTCTACTACGAGGCG R: TGCTCTCCGATGCTACCCA	NM_176788.1
FABP4	F: GTTTGAATGGGGGTGTGGTC R:CGATGCTCTTGACTTTCCTGTC	NM_174314.2
PPARG	F: TGGCCATTGAATGCCGGGTC R: ACATCCCCACAGCAAGGCAC	NM_181024.2
TGFB	F: AACCTGTGTTGCTCTCTCGG R: GAGGTAGCGCCAGGAATTGT	NM_001166068.1
Zfp423	F: GGATTCCTCCGTGACAGCA R: TCGTCCTCATTCCTCTCCTCT	NM_001101893.1
18S	F: CCTGCGGCTTAATTTGACTC R: AACTAAGAACGGCCATGCAC	NR_036642.1

For PPARG and FABP4, we also performed Western Blotting analysis, using Beta-actin (β-actin) as the endogenous control. Cells were collected from the culture dishes with 1mL of lysis buffer (urea 7M, thiurea 2M, CHAPS 4%, dithiothreitol 1%, IPG buffer 2%, protease inhibitor cocktail 1M) per well. Protein content was separated through 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked with blocking solution (3% BSA w/v in Tris-Buffered Saline and Tween 20,

(TBSt)) for 1 hour with gentle agitation at room temperature. Membranes were then incubated with the following primary antibodies against PPARG, FABP4 and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). Primary antibodies were incubated at 1:500 dilution in the blocking solution for 16 h at 4°C with gentle agitation. After incubation with primary antibodies, membranes were washed 3 times at room temperature with TBSt and then incubated for 1 hour with the appropriate horseradish peroxidase secondary antibody at 1:8,000. Then, membranes were washed 3 times (5 min each) with TBSt, scanned and analyzed with ImageJ.

3. Animals and sampling

In order to investigate the *in vivo* effect of RA on adipogenesis in cattle skeletal muscle, we conducted an experiment using animals (n=12) obtained from a crossing (50%Wagyu, 25% Angus and 25% Nellore). All animals were castrated male, siblings from the same father, raised under the same grazing conditions, weaned at eight months of age and submitted to the finishing diet as seen in Table 2. The treatment consisted of supplementation or not with vitamin A (six of them received vitamin A supplementation and six of them were in control group) and maintained in the same conditions for 320 days in feedlot. After that, animals were slaughtered with an average of 19 months of age, all of them belonged to a group with accepted marbling standard for exportation.

Table 2 - Ingredient composition of the experimental diets.

Item	Non-supplemented with Vitamin A	Supplemented with Vitamin A
Ingredient composition (% DM)		
Corn silage	20.0	20.0
Corn grain	40.3	40.3
Soybean hull	16.0	16.0
Cottonseed cake	15.7	15.7
Soy molasses	4.5	4.5
ProteN[1]	1.0	1.0
Mineral Mixture without Vit A[2]	2.5	-
Mineral Mixture with Vit A[3]	-	2.5

[1]Rumen protected urea with 40.32% of N;

[2]Ca = 22.8%; P = 2.4%; Mg = 2.0; Na = 8.1%; S = 8.1%; Fe = 588 mg/kg; Cu = 504 mg/kg; Zn = 2400 mg/kg; Mn = 1008 mg/kg; Se = 8.4 mg/kg; Co = 30.2 mg/kg; I = 30.2 mg/kg; Vitamin D3 = 12.49 KUI/kg; Vitamin E = 291.5 mg/kg; Monensin = 1000.2 mg/kg;

[3]Ca = 22.8%; P = 2.4%; Mg = 2.0; Na = 8.1%; S = 8.1%; Fe = 588 mg/kg; Cu = 504 mg/kg; Zn = 2400 mg/kg; Mn = 1008 mg/kg; Se = 8.4 mg/kg; Co = 30.2 mg/kg; I = 30.2 mg/kg; Vitamin A = 99.9 KUI/kg; Vitamin D3 = 12.49 KUI/kg; Vitamin E = 291.5 mg/kg; Monensin = 1000.2 mg/kg

The slaughter and samples collection were conducted in a commercial slaughtering house in Lins/SP. At slaughter, 50 grams of skeletal muscular tissue were collected from the *Longissimus dorsi* muscle, located at the level of the 10th and 11th ribs. All instruments used for the collection were sterilized in order to avoid contamination. The samples were stored in liquid nitrogen and transported to the Animal Biotechnology Laboratory (LABTEC) at Universidade Federal de Viçosa – UFV, Viçosa/MG for further analyses. Samples were processed and stored at -80°C to prevent degradation.

4. Two-dimensional gel electrophoresis

For protein extraction, 500mg of muscular tissue were used in 1mL of lysis buffer (urea 7M, thiurea 2M, CHAPS 4%, dithiothreitol 1%, IPG buffer 2%, protease inhibitor cocktail 1M). The protein content of the samples was measured according to Bradford (1976). Samples were solubilized in rehydration solution, IPG strips of 24cm, pH 3-10, and then rehydrated with 450μL of protein solution for 10 hours in Immobiline DryStrip Reswelling (GE Healthcare, Little Chalfont-UK) tray. After that, the isoelectric focalization was performed using Ettan IPGphor III System (GE Healthcare, Little Chalfont-UK) at 20°C, followed by the balance of the strips, using dithiothreitol and iodoacetamide solutions. Samples were submitted to molecular weight separation in 12.5% SDS-PAGE gels, using Ettan DaltSix (GE Healthcare, Little Chalfont-UK) apparatus (cycle 1: 80V for 45 minutes and cycle 2: 500V for 8 hours).

5. Gel Staining and Spot Analysis

Immediately after running, SDS-PAGE gels were fixed (methanol 50% and acetic acid 10%) and stained with Pro-Q® Diamond Phosphoprotein Gel Stain (200 mL/gel) for phosphorylated proteins. Gels were washed (acetonitrile, sodium acetate and distilled water) followed by scanning with Fujifilm FLA-5100 (FUJIFIL Life Science, Stanford-USA). After that, gels were stained with SYPRO® Ruby Protein Gel Stain (200 mL/gel) for total proteins and kept at room temperature with gentle agitation overnight. After that gels were destained (methanol 10% and acetic acid 7%) and scanned. For visualization and determination of differentially expressed spots, gels were stained with Coomassie

Blue G250 Gel Stain. The image analysis of the gels was carried out on ImageMaster 2D Platinum Software 5.0 (GE Health Care, Little Chalfont-UK). The relative spot volumes were determined by normalising to all spots in the gel. Briefly, all gel images were processed and analyzed under the same parameters and, after spot detection, automatically matched with the spots of a master gel used as a reference. Landmark spots were used to confirm spot matching across all gels and manual verification was used to screen out any dust artifacts or incorrectly identified spots.

6. Tryptic Digestion of Spots and Peptides Identification

The differential spots were cut from the SDS-PAGE gels and treated with destaining solution (10 μL of ACN/100 mM ammonium bicarbonate 1:1 v/v). The protein disulphide bonds were reduced by the addition of dithiothreitol (65mM) and alkylated by iodoacetamide (200mM). Each spot was dehydrated in acetonitrile (ACN) (Sigma-Aldrich, St.Louis, Missouri-USA). Trypsin (Promega, Fitchburg-USA) (1 μg/μL in 50 mM acetic acid) was added and the digestion allowed to proceed overnight at 37°C. The peptides were then extracted from the gel by addition of ACN solution (ACN50%, formic acid 5% and distilled water) and then dried overnight in the equipment Vacufuge (Eppendorf, Hamburg-Germany). Samples were desalinated in hydrophobicity column C18 Zip Tip (Eppendorf, Hamburg-Germany) following the manufacturer's instructions and 1 μL of each sample containing the peptides was applied in a steel plate for mass spectrometer coupled with matrix of acid α-cyano-4-hidroxilcinamico (Sigma-Aldrich, St.Louis, Missouri-USA) in a 1: 1 ratio.

Mass spectrometry MALDI TOF/TOF was used for identification of peptides in order to establish the protein profiling accross the animals from both treatments. The mass analysis of the peptide mix was carried out on an Ultra flex MALDI-TOF/TOF with a lift module (Bruker Daltonics). The acquired MS/MS spectra were transformed in mz.data format and used for protein identification with Mascot 2.1 (Matrix Science, Boston, MA). Protein identification was made using the MASCOT version 2.2 software (Matrix Science, Boston, MA, USA) at the MS/MS ion search mode, with the following parameters: tryptic specificity, one missed cleavage, and a mass measurement tolerance of 0.2 Da for MS and 0.5 Da for MS/MS mode. Cysteine carbamidomethylation was used as fixed modification, while methionine oxidation was used as variable modification. The database used was the Bovidae deposited in UniProt (http://www.uniprot.org/). The software SCAFFOLD (Proteome Software, Portland, Oregon-USA) was used to validate the proteins, using the following parameters: 95% of probability, 1 as the minimum number of peptides.

7. Statistical analysis

Statistical analysis for gene expression was performed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) followed by a t-test to compare the relative expression between the treatments. Regarding Western Blotting analysis, bands were compared using ImageJ software. For the protein analysis, ImageMaster 2D Platinum Software (GE Health Care, Little Chalfont-UK) was used to quantify the spots using the % of spot volume criterion, which is automatically calculated. The match analysis was performed in an automatic mode and further manual editing was performed to correct mismatched and unmatched

spots. A criterion of P< 0.05 was used to define the significant difference when analyzing the paired spots between the treatments according to ANOVA.

RESULTS

In the present study, we identified the effect of RA on the commitment of SV 5-Aza-2′-deoxycytidine treated cells to the adipogenic lineage. Differential expression of *FABP4* and *Zfp423* was identified (P=0.008 and P=0.02, respectively), indicating that RA decreased the gene expression. No differences (P>0.05) were observed for *CEBPA* (0.92), *CEBPB* (0.64), *PPARG* (0.26) and *TGFB* (0.93).

For PPARG and FABP4, we performed Western Blotting analysis, using Beta-actin (*β-actin*) as the endogenous control, and we found a significant difference (P=0.008) between treatments, where RA decreased the FABP4 protein content, as indicated by darker bands in the control group against the lighter/clearer bands in RA group (Figure 1). For PPARG we did not find any difference, as we can see in Figure 1.

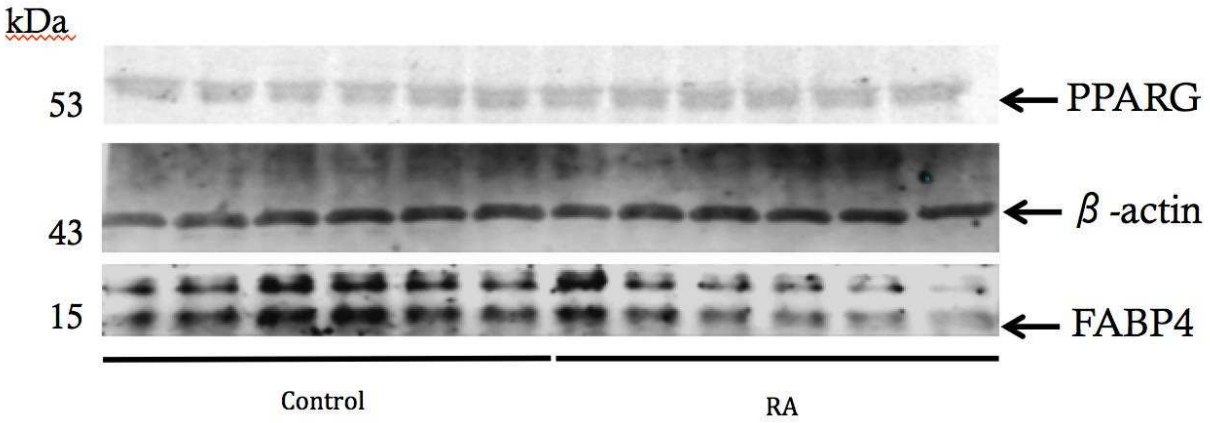

Figure 1. Western Blotting for PPARG and FABP4, using β-actin as endogenous control. Control= cells not treated with retinoic acid, RA= cells treated with retinoic acid.

The results of Western Blotting analysis are consistent with gene expression results, showing that RA decreased the gene expression of *ZFP423* and *FABP4* and protein content of FABP4.

In the present study, we also investigated protein composition of *Longissimus dorsi* muscle samples from animals supplemented or not with vitamin A. Proteomics analyses (2-DE and MS/MS identification) yielded ten differentially expressed spots ($P<0.05$) upon comparison of 12 gels (six per treatment), which were identified via MS/MS and validated by SCAFFOLD software, as we can see in Table 2.

Table 2– Identification of proteins extracted from bovine muscle skeletal tissue of animals supplemented or not with Vitamin A, identified by MALDI TOF/TOF, MASCOT algorithm and SCAFFOLD software against Bovidae database from Uniprot.

Spot	Protein Name/Organism	Database	Acession Number	Score of Identification (MS/MS)	Prob.(a)	Sequence Coverage	Theoretical MW	Theoretical pI	Experimental MW	Experimental pI	NPIV(c)	Prob.(b)
1	Troponin C	Bovidae/Uniprot	Q148C2	650	100%	50%	18kDa	4.06	15.8kDa	3.43	8	99%
5	Actin alpha 1	Bovidae/Uniprot	A4IFM8	116	100%	29%	42kDa	5.23	52kDa	4.56	8	97%
5	Tropomodulin	Bovidae/Uniprot	L8HY73_9CETA	65	95%	3%	40kDa	5.07	52kDa	4.56	1	98%
6	Actin alpha 1	Bovidae/Uniprot	A4IFM8	777	99%	3%	42kDa	5.23	45kDa	5.42	1	95%
7	Actin alpha 1	Bovidae/Uniprot	A4IFM8	547	100%	22%	42kDa	5.23	43kDa	5.58	8	100%
10	Malate dehydrogenase	Bovidae/Uniprot	D4QC3_BUBBU	78	99%	4%	36kDa	7.7	42kDa	9.6	1	97%
18	Desmin	Bovidae/Uniprot	DESM_BOVIN	475	100%	18%	54kDa	5.21	63kDa	5.22	6	96%
19	ATP synthase subunit beta	Bovidae/Uniprot	ATP5B	696	100%	22%	56kDa	5.2	62kDa	5.1	9	99%
604	ATP synthase subunit beta	Bovidae/Uniprot	ATP5B	523	100%	20%	56kDa	5.15	47.9kDa	5.1	7	97%
605	L-lactate dehydrogenase	Bovidae/Uniprot	A0FH35	111*	*	*	37kDa	*	26.9kDa	6.5	*	*

Legend:
(a) Probability of identification for proteins (Scaffold)
(b) Probability of identification for peptide (Scaffold)
(c) NPIV – Number of peptides identified and validated (Scaffold)
MW: Molecular weight
pI: isoelectric point
*Protein identified only by Peptide Mass Fingerprinting.

DISCUSSION

Adipogenesis is the process of proliferation and differentiation of preadipocytes, which involves a cascade of transcription factors and cell-cycle proteins regulating gene expression and leading to adipocyte development (Moreno-Navarrete and Fernández-Real, 2012). It is influenced by many factors as expression of particular adipogenic genes and transcription factors. We can highlight two of them, studied in this experiment, *FABP4* and *Zfp423*. *FABP4* is an important transcription factor playing a role during adipogenesis, acting as a fatty acids chaperone, which couples intracellular lipids to biological targets and signaling pathways (Garin-Shkolnik et al., 2014). *Zfp423* is known as a key initiator of adipogenic differentiation, being a critical regulator of the adipogenic potential of bovine SV cells (Huang et al., 2012) and recently, based on studies in mice, it was also identified as a transcriptional factor responsible for the adipogenic commitment of progenitor cells (Gupta et al., 2010).

Retinoic acid (RA) is a metabolite of vitamin A that affects progenitor cells and mature adipocytes differently due to the stage-specific expression of related transcription factors (Wang et al., 2016), and relates to the pathway of RAR (retinoic acid receptors) or by PPAR pathway. RA is a potent inhibitor of adipogenesis, and its action appears to block *CEBPB* transcriptional potential during early differentiation (Marchildon et al., 2010).

Some studies have reported that RA promotes adipogenic commitment of progenitor cells and encumber terminal differentiation of adipocytes. In our experiment

we did not use embryonic cells, so we used a DNA-demethylation agent, trying to return all cells to a progenitor cells stage and reduce the epigenetic marks that could interfere in the adipogenesis, since the promotion of retinoic acid on adipogenic commitment involves epigenetic modifications (Wang et al., 2016).

As a transcriptionally controlled process, adipogenesis is accompanied by several histone modifications. In particular, histone H3 and H4 acetylation is associated with the activation of adipogenic gene expression (Debril et al., 2004). So, the decreased commitment of cells to adipogenic lineage seen by the lower expression of *Zfp423* and *FABP4* genes could be explained by the fact that some other modifications in the DNA, as histone modifications, can be related to the commitment of the cells with the adipogenic lineage. We demonstrated that RA plays a distinct metabolic role in adipocyte differentiation *in vitro*, inhibiting some of adipogenic key genes expression. Up to now, in livestock cells, epigenetic changes in these genes during adipogenic differentiation have not been examined and warrant further studies. According to Wang et al. (2016), there are many evidences supporting the inhibitory roles of methyl donor supplementation in suppressing adipogenesis both *in vivo* and *in vitro*, such as folate supplementation which increases overall CpG methylation in *CEBPA* promoter, and inhibits its expression and adipogenesis in chicken preadipocytes (Yu et al., 2014).

Regarding to the *in vivo* experiment, overexpressed proteins in *Longissimus dorsi* muscle from animals non-supplemented with vitamin A mainly belonged to (i) energy metabolism – L-lactate Dehydrogenase (LDHA), Malate dehydrogenase (MDHA), (ii) ATP synthesis - ATP synthase subunit beta (ATP5B), (iii) structural muscle proteins – Actin alpha 1 (ACTA1) and Tropomodulin (TMOD).

The higher abundance of LDH protein in muscle from animals non-supplemented with vitamin A can be explained by the fact that LDH has been reported to increase its expression in a model of adipogenesis induced through peroxisome proliferator-activated receptor gamma (*PPARG*) activation (Mueller et al., 2002). Nonetheless, this is not totally unexpected, as previous observations on high fat deposition breeds did not show to correlate with *PPARG* expression changes. Hence, we can infer that these animals had an increased fat deposition, compared to animals supplemented with vitamin A. LDH converts pyruvate to lactate, which is the second route (glycolytic pathway) for lactate metabolism, and indicates the anaerobic-glycolytic capacity in muscles and consequently lactate accumulation resulting in ATP consumption and, subsequently, accumulation of AMP (activation of AMPK and autophagic pathways), alteration of ion homeostasis modulation (blockade of ion pumps and calcium release from mitochondria and sarcoplasmic reticulum) (D'alessandro and Zolla, 2013). Calcium intracellular accumulation activates calpains, which will promote apoptosis through the mitochondrial intrinsic pathway to tenderization of the muscle through apoptosis.

Similarly, the increased content of MDHA observed in animals non-supplemented with vitamin A, might reflect oxidative metabolism, and leads to an increase of conversion of malate to oxaloacetate producing an equivalent amount of NADH (Bax et al., 2013) and increasing the formation of acetyl-CoA, the main fuel for the tricarboxylic acid cycle and formation of fatty acids.

ATP synthase subunit beta plays a key role in oxidative metabolism and fat content in skeletal muscle (Xu et al., 2013), so elevated ATP levels can also be achieved by the up-regulation expression of this enzyme. Mitochondrial ATP synthase catalyzes

ATP formation, utilizing a proton electrochemical gradient through the inner membrane during oxidative phosphorylation. According to Xu et al. (2013), the high expression of ATP5B in Meishan *Thorax-waist Longissimus dorsi* muscle indicated that this breed possesses greater oxidative capacity than the Large White breed. In skeletal muscle, the greater fat content means more oxidative metabolism to increase energy metabolism (Hocquette et al., 1998), which is predominant in slow twitch muscle fibers. In these fibers, oxidative metabolism is one of the main pathways through which the animal derives energy for muscle contraction and to fuel energy demands for growth, while the glycolysis pathway is predominant in fast twitch muscle. The results observed in this study, show that one phosphorylated isoform (revealed in Pro-Q® Diamond staining) of ATP5B was more abundant in muscle from animals supplemented with vitamin A, while another isoform of ATP5B (revealed in Coomassie staining) was more abundant in muscle from non-supplemented animals. As phosphorylation can either activate or inactive a protein, in this case we can imply that the protein was inactive, suggesting AMP accumulation and subsequent use of this molecule in fatty acid synthesis, increasing fat deposition in muscle from non-supplemented animals, leading them to an oxidative metabolism.

Regarding to structural proteins, two phosphorylated isoforms (revealed in Pro-Q® Diamond staining) of ACTA1 were found to be overexpressed in muscle from vitamin A supplemented animals, and one non-phosphorylated isoform (revealed in Coomassie staining) more abundant in muscle from non-supplemented animals. It is known that alpha-actin positively correlates with synthesis of muscle fiber proteins and, ultimately, with muscle growth. Approximately 50% of the protein content of the muscle

fiber is made up of the contractile machinery, mostly consisting of myosin complexes of the thick filaments and actin strings of the thin filaments (Murgiano et al., 2010). The upregulation of ACTA1 in vitamin A supplemented animals is completely coherent with the greater tendency to mass accumulation, instead of fat deposition. With respect to Tropomodulin, which is an end actin filament capping, the results show an overexpression of a phosphorylated isoform in addition to the overexpressed isoform of ACTA1 in muscle from animals non-supplemented with vitamin A. Also, it suggests that different isoforms of such structural proteins are required for muscle development and muscle fibers formation.

Conversely, proteins which were observed to be overexpressed in *Longissimus dorsi* from vitamin A supplemented animals, mainly accounted for (i) structural muscle proteins - Actin alpha 1 (ACTA1) where two different isoforms were identified, Desmin (DESM) and Troponin C (TNNC), (ii) ATP synthesis – ATP synthase subunit beta (ATP5B).

The troponin complex is pivotal to Ca^{2+} regulation of muscle contraction. Troponin consists in three subunits: the Ca^{2+} binding protein, Troponin C (TNNC) or TnC, the inhibitory subunit, Troponin I or TnI, and the tropomyosin binding subunit, Troponin T or TnT (Bax et al., 2013). Troponin C is required for muscle contraction by ligation of Ca^{2+} on its binding site, which will allow myosin to couple actin, resulting in contraction. Hence the greater contraction, the greater chances of toughness, so the higher amount of TNNC observed for muscles in vitamin A supplemented animals indicates increased probability of low meat quality (Lana and Zolla, 2015).

Considering DESM, another structural protein important to the muscle ultrastructure (Lomiwes et al., 2014), the results show that its phosphorylated isoform was more abundant in muscle from supplemented animals, as well as other overexpressed structural proteins increasing muscle fiber mass, unlikely intramuscular fat content. Therefore, reduction of vitamin A intake has been used to enhance intramuscular lipid accumulation and marbling in finishing beef cattle (Arnett et al., 2009). In this way, feeding no supplemental vitamin A has potential to optimize carcass quality and cutability. Summarizing, dietary supplementation can be further studied, looking for the mechanisms by which the additive will affect, depending on the goal for the final product, as increasing muscle growth or increasing fat deposition.

CONCLUSION

The present findings in the *in vitro* and Western Blotting studies for RA treated cells comparing to cells that did not received RA, showed the RA effect on decreasing gene expression of *ZFP423* and *FABP4* and protein content of FABP4. These results were used as a background for the *in vivo* proteomic study, which was aimed at detecting representative proteins. Most of the differentially expressed proteins in non-supplemented animals which have been proposed to play a role in fatty acids formation and lipid accumulation. In supplemented animals, we observed the presence of proteins related to muscle fiber structure, clearly showing a tendency to muscular growth owing to the fact that most of the overexpressed proteins belonged to protein groups composing the fiber and the sarcoplasmic reticulum, as well as with enzymes. The results of *in vitro* study are

in accordance with the *in vivo* results, showing the decreased fat deposition affected by vitamin A supplementation. In fact, there is a significant difference in the commitment of cells to adipogenic lineage, when they are treated with retinoic acid *in vitro* and also a difference in fat deposition *in vivo* when using supplementation with vitamin A. If the goal is to increase the intramuscular fat in carcass, it is essential to control the levels of dietary vitamin A. Concluding, the present study contributed to a better understanding of several mechanisms explaining adipogenesis.

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CHAPTER V

GENERAL DISCUSSION

Introduction

Marbling or intramuscular fat (IMF) has been considered one of the top beef quality problems (Du et al., 2013). In this way, many studies have been conducted over the last years, seeking to understand the mechanisms underlying adipogenesis, the process by which cells differentiate into mature adipocytes and start to accumulate lipids. These studies became essential to make possible to manipulate the whole process to increase meat quality. In this chapter, we discuss the importance of *in vitro* studies to elucidate genes and transcription factors involved in adipogenesis (chapters III and IV). Moreover, an *in vivo* study regarding differential protein profiles influenced by supplementation with vitamin A (chapter IV) is also discussed.

Genetic divergences

In the previous chapters we have seen significant differences both in cattle and in pigs regarding to IMF, including previous studies which suggest that the high deposition of IMF in Wagyu cattle is related to the differential expression of specific genes (Duarte et al., 2013) in relation to other cattle breeds. So, we highlighted the Wagyu beef cattle as a model for the studies of adipose tissue deposition on chapter II. In addition, we pointed out differences on the expression of genes and transcription factors between two genetic groups, a commercial pig line and Piau breed, as revealed by *in vitro* induction with

adipogenic protocol. Adiponectin (*ADIPOQ*), CAAT/Enhancer Binding Protein alpha (*CEBPA*) and Stearoyl-CoA Desaturase 1 (*SCD1*) were overexpressed in stromal vascular cells from the commercial pig line, compared to Piau origin cells. These results (chapter III) indicate a higher accumulation of lipids by these cells, which we could visualize by staining the cells with the Oil Red-O and the visualization of red small dots inside the culture, in accordance with Sollero et al. (2011), who reported important differences on gene expression between the same genetic groups.

Depending on origin, cells isolated and cultured *in vitro* can present atypical patterns of expression, presenting different responses. The gene expression study indicated that cells from commercial pig line behave differentially when submitted to an induction of adipogenic protocol in relation to Piau breed cells, showing an increased ability to differentiation of preadipocytes into mature adipocytes and lipid accumulation. This enlightenment is meaningful to establish crossbreeds, taking into account the complementarity between breeds, depending on the goal for the final product, determining the increase or decrease of fat deposition on meat.

Feeding additives and effects on meat quality

Many factors can influence *in vitro* and *in vivo* adipogenesis, so in another study (Chapter IV), we conducted an *in vitro* and a Western Blotting study using stromal vascular cattle cells aiming to reveal the effect of retinoic acid (RA), a metabolite of vitamin A, in the commitment of cells to adipogenic lineage. The results showed the decreased expression of transcription factors: Fatty acid binding protein 4 (*FABP4*) and

Zinc finger protein 423 (*Zfp423*) and also a decrease on FABP4 protein content, indicating that RA reduces adipogenesis. In order to investigate RA effect *in vivo,* we performed a proteomic experiment.

One of the major goals of proteomics in the field of farm animal science is to shed light on skeletal muscle biochemistry (Picard et al., 2010) and to deepen our understanding of the physiological changes taking place at the protein level following harvest (D'alessandro and Zolla, 2013). In this study, we compared the protein profiles of cattle supplemented or not with vitamin A, and results indicated that vitamin A supplemented animals showed higher abundance of structural proteins while proteins of non-supplemented animals mainly belonged to energy metabolism.

These findings demonstrate the need for a deeper biological understanding of mechanisms underlying intramuscular fat deposition. Since retinoic acid is derived from vitamin A and its use leads to the gene expression involving lipolysis and decreased lipid content, reducing vitamin A dietary levels could promote lipid accumulation. Furthermore, vitamin A supplementation can imply differential protein profiles of skeletal muscle and the elucidation of effects become crucial to generate knowledge that can be used to modify marbling levels on carcasses.

Concluding remarks

The gene expression *in vitro* studies provide a rich information about genes and transcription factors involved in adipogenic differentiation, comparing a commercial pig line and Piau pig breed, showing time-dependence and breed-specific genes. For cattle cells, we observed the effects of retinoic acid treatment in expression of adipogenic trancription factors and protein content. Moreover, the proteomic *in vivo* study reported the effects of vitamin A on proteomic profiles in skeletal muscle in cattle, displaying differentially expressed proteins between treatments. Thereby, we provided a greater understanding of regulatory mechanisms under a different way of manipulation of intramuscular fat in livestock animals.

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