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Integrative analysis of microRNAs and mRNAs involved in regulation of intramuscular fat deposition in Nelore cattle

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DEDICATION

I dedicate this work to my father Cláudio and my mother Ana Beatriz for all support, encouragement, patience and love during my master studies and during all my life.

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“Quem vence sem risco, triunfa sem glória...
Não tenha medo da vida, não tenha medo de vivê-la.”.

Augusto Cury.

SUMMARY

RESUMO

Análise de integração de dados de microRNAs e mRNAs envolvidos na regulação da deposição de gordura intramuscular em bovinos Nelore

A quantidade de gordura intramuscular pode influenciar as características sensoriais e o valor nutricional da carne bovina, assim, a seleção de animais com conteúdo de gordura adequado para o consumidor torna-se importante. A gordura intramuscular é uma característica complexa, de difícil medição e há um conhecimento crescente sobre os genes e vias que controlam os processos biológicos envolvidos na deposição de gordura no músculo. MicroRNAs (miRNAs) são uma classe bem conservados de pequenos RNAs não-codificantes, que modulam a expressão gênica de uma gama de funções no desenvolvimento e fisiologia animal. Este estudo objetivou identificar miRNAs diferencialmente expressos (DE), genes reguladores candidatos e redes de co-expressão usando dados de expressão de mRNAs e miRNAs do músculo *Longissimus dorsi* de 30 novilhos Nelore com valores genéticos genômicos estimados (GEBV) extremos para conteúdo de gordura intramuscular (IMF). A análise de expressão diferencial entre os dados de miRNA de animais com valores extremos de GEBV para o IMF identificou seis miRNAs DE. A anotação funcional de genes alvos destes microRNAs indica que a via de sinalização de PPAR está envolvida com a deposição de IMF. Os genes reguladores candidatos, tais como SDHAF4, FBXO17, ALDOA e PKM foram identificados pelas abordagens de correlação parcial com teoria da informação (PCIT), fator de impacto fenotípico (PIF) e fator de impacto regulatório (RIF) a partir de dados integrados de expressão de mRNAs-miRNAs. Dois miRNAs, bta-miR-143 e bta-miR-146b, com alta expressão no grupo de baixo conteúdo de IMF, também foram correlacionados com genes reguladores candidatos, os quais foram funcionalmente enriquecidos para termos GO relacionados a oxidação de ácidos graxos. As redes de co-expressão identificaram vários módulos relacionados ao sistema imunológico, ao metabolismo das proteínas, ao metabolismo energético e ao catabolismo da glicose através da análise ponderada da rede de correlação (WGCNA), que mostrou possível interação e regulação entre mRNAs e miRNAs. Este estudo contribui com a compreensão dos possíveis mecanismos reguladores das redes de sinalização genética envolvidas no processo de deposição de gordura. O metabolismo da glicose e o processo de inflamação foram as principais vias encontrados na análise integrada de mRNA-miRNA e mostraram estar associadas ao conteúdo de gordura intramuscular em bovinos de corte.

Palavras-chave: Lipídeos; RNA-Seq; *Bos indicus*; MicroRNAs; Redes de co-expressão

ABSTRACT

Integrative analysis of microRNAs and mRNAs involved in regulation of intramuscular fat deposition in Nelore cattle

The amount of intramuscular fat can influence the sensory characteristics and nutritional value of beef, thus the selection of animals with adequate fat content for consumer becomes important. Intramuscular fat is a complex trait that is difficult to measure and there is growing knowledge about the genes and pathways that control the biological processes involved in fat deposition in muscle. MicroRNAs (miRNAs) are well conserved class of non-coding small RNAs that modulate gene expression of a range of functions in animal development and physiology. This study aimed to identify differentially expressed (DE) miRNAs, regulatory candidate genes and co-expression networks using mRNAs and miRNAs expression data from the *Longissimus dorsi* muscle of 30 Nelore steers with extreme genomic estimated breeding values (GEBV) for intramuscular fat (IMF) content. The differential expression analysis between the miRNA data from animals with extreme GEBV values for IMF identified six DE miRNAs. Functional annotation of target genes for these microRNAs indicates that PPARs signaling pathway is involved with IMF deposition. Regulatory candidate genes such as SDHAF4, FBXO17, ALDOA and PKM were identified by partial correlation with information theory (PCIT), phenotypic impact factor (PIF) and regulatory impact factor (RIF) approaches from integrated miRNAs-mRNAs expression data. Two DE miRNAs, bta-miR-143 and bta-miR-146b, upregulated in Low IMF group, were also correlated with regulatory candidate genes, which were functionally enriched for GO terms for fatty acids oxidation. Co-expression networks identified several modules related to immune system, protein metabolism, energy metabolism and glucose catabolism by weighted correlation network analysis (WGCNA), which showed possible interaction and regulation between mRNAs and miRNAs. This study contributes to our understanding of regulatory mechanisms of gene signaling networks involved in fat deposition process. Glucose metabolism and inflammation process were the main pathways found in integrative mRNAs-miRNAs analysis and showed to influence intramuscular fat content in beef cattle.

Keywords: Lipids; RNA-Seq; *Bos indicus*; MicroRNAs; Co-expression networks

1 INTRODUCTION

Brazil has the largest commercial cattle herd in the world with over 219 million head in 2016, 48 million head slaughtered and from a total of 9,284 tons, according to United States Department of Agriculture [1].

In Brazil, beef cattle herd are mostly Nelore breed (*Bos taurus indicus*) or their crossbreeds. The predominance of this breed is due to the greater productivity of these animals at tropical conditions, because they are resistant to heat and parasites [2]. Other important characteristic of the zebu animals is that when they are slaughtered in earlier ages, in relation to taurine animals, present more homogeneous distribution of subcutaneous fat (coverage) and lower amount of intramuscular fat [3]. Characteristic that for some markets such as European (continental) the least amount of fat in the carcass is desirable, but for others, like Asian and British this feature makes Brazilian beef with lower market value [4].

Despite the prominent position of the beef cattle industry in the economic scenario, Brazil still exports meat without added value to the international market. Thus, the control of beef quality, and their sensory characteristics (tenderness, flavour, juiciness and colour) are very important [5]. Meat quality depends on many factors and the amount of intramuscular fat (IMF) affects the sensory characteristics and nutritional value of meat, which is an important parameter to ensure a more juicy and tasty meat [6].

The genetic component shows to be highly crucial for several traits affecting meat quality. Experiments comparing the transcriptional profile among breeds with different potential for intramuscular fat deposition provide important information about differentially expressed genes that may be able to be used as candidate genes in beef cattle breeding programs [7]. Thus the understanding of genes regulation associated with adipose tissue growth and lipid metabolism becomes important to identification of potential regulators of phenotype.

1.1 Gene regulation of lipid metabolism

The control and organization of cellular and physiological responses to different metabolic conditions occurs at the level of gene regulation in the nucleus. Many key regulators of gene expression, such as transcription factors or non-coding RNAs, may respond directly or indirectly to metabolic signals such as lipids, glucose, and insulin, rapidly altering gene pathways responsible for metabolic homeostasis [8]. The regulation of lipid homeostasis is mediated by the influence of nutritional status, energy expenditure, hormonal physiological response, enzymatic activity and molecular mechanisms, which promote the modulation of adipogenesis and lipogenesis [9-11].

Lipids are important metabolites in the body, participating as cellular components of membranes, important for energy generation and also act as signaling molecules in physiological processes [8,10]. The white adipose tissue is the main source of lipids of the organism, storing them in the form of triacylglycerols (TAGs) in the adipocytes, which serve as energy storage. Besides the adipocytes tissue have other important cellular structures for its maintenance as preadipocytes (adipocyte precursor cells), endothelial cells, vascular stroma cells, fibroblasts, leukocytes, and macrophages. Modifications in adipocyte size (hypertrophy) and number (hyperplasia) of cells occur in response to activation of metabolic functions that react according to the need of adipocytes differentiation, incorporation or release of lipids [9,10].

The differentiation of the adipocytes consists of stages controlled by the activation of genes previously silenced, initiating the process of adipogenesis. Activation of binding proteins to the CCAAT (CCAAT/enhancer binding proteins, C/EBPs), known as C/EBP-b and C/EBP-s, occurs first. The cells then enter in terminal differentiation by activation of the PPARγ (from the peroxisome proliferator-activated receptors family) and C/EBP-a, the two central regulators of the adipogenic process [12]. These regulators remain active through positive feedback until the moment of cell compromise in differentiation, where other target genes are already being activated to maintain this process, such as enzymes and proteins involved in the transport of glucose, lipogenesis, lipolysis and adipokine synthesis and secretion [9]. After the formation of this adipocyte structure, begins the incorporation and storage of lipids, originated from the synthesis process during lipogenesis. Lipogenesis is the process by which metabolism intermediates are converted to fatty acids, which can be incorporated into triacylglycerols for tissue energy storage. During fasting, the opposite occurs and the lipid degradation pathway known as lipolysis is activated, so the TAGs of the adipose tissue are hydrolyzed by releasing free fatty acids that can be recovered by the body and used as a source of energy for organism [10].

These processes are controlled by genetic mechanisms that can activate or inhibit genes and enzymes of lipogenic and lipolytic pathways [13]. The genes PGC-1β and PGC-1α, members of the transcriptional co-activators PGC-1 family (peroxisome proliferator-activated receptor-gamma coactivator), have been described to increase the fatty acid oxidation, through the coactivation of PPARα [14, 15]. However, it has been shown that the overexpression of PGC-1β induces the transcription of several lipogenic enzymes involved in lipogenesis [16], through the activation of other signaling pathways, showing the different functions of the same gene according to stimulus received.

Another important gene is the LXR (liver X receptors), a member of the superfamily of nuclear receptors that heterodimerize with the RXR (retinoid X receptor). This LXR gene plays a role in the lipid and cholesterol metabolism [17] by indirectly activating lipogenic genes through the activation of the SREBP-1c transcription factor (from the Sterol regulatory element-binding proteins family) and from ChREBP (carbohydrate-responsive element-binding protein), both involved in the activation of glucose pathway enzyme genes and fatty acid synthesis [13].

PPARs can also acts as transcription factors heterodimerizing with RXRs and binding to specific DNA regions of target genes. PPAR's family is formed by PPARα, PPARγ and PPARβ, which each one presents a specific have different function in lipid metabolism. PPARα and PPARβ show their expression level stimulated during fasting and influenced by some hormones, such as growth, leptin and insulin [18]. They are related to lipolysis, i.e. in the oxidation of fatty acids, mainly described in skeletal muscle where their expression is higher [19, 20]. On the other hand, PPARγ is mainly important for adipogenesis, promoting the proliferation and differentiation of adipocytes and also improve insulin sensitivity through increased liver and muscle glucose uptake and reduced circulation in the bloodstream [21].

1.2 microRNAs regulation

The emergence of RNA sequencing (RNA-seq), for gene expression study, using NGS technologies (Next Generation Sequencing) has allowed the access to transcriptome analysis [22]. This technology also provides a more accurate measure of transcript levels compared to microarray technology [23] and the expression analysis of small RNAs, such as microRNAs (miRNAs), has also been improved.

The regulation of the expression of genes that control lipid metabolism has many levels and can be regulated by different molecular mechanisms. One of these regulatory steps is by modulating gene expression through non-coding RNAs such as miRNAs.

MiRNAs are endogenous ribonucleic acids (RNAs), non-coding (ncRNA) of approximately twenty-two nucleotides in length [24]. The first miRNA was discovered in 1993 in *Caenorhabditis elegans* and was called lin-4 to be related to suppress a gene encoding the lin-4 protein, which has activity in the larval development of this nematode species [25]. In mammals, miRNAs perform their regulatory effect at post-transcriptional level in many tissues and are associated with the control of several important biological processes related with lipid metabolism [26]. This regulatory effect is mainly through imperfect complementarity between the miRNA and the 3'UTR region of the target mRNA, promoting the inhibition of translation [27, 28]. In addition, the miRNAs affect directly the translation initiation factors, disrupting poly-A tail function [29], and consequently causing a reduction in the protein levels of their target genes [30]. Because miRNAs have small sequences and act without complete pairing, a single miRNA can regulate many target mRNAs, in addition to cooperating in the control of a single mRNA [31]. Thus, miRNAs constitute a complex regulatory network of cell signaling [32], and can act directly on the mRNA transcript or indirectly through the regulation of intermediary components that influence the expression of genes, such as transcription factors. Besides that, miRNAs act on coactivating genes present in signaling networks that control the expression of transcription factors, thus forming an additional layer of indirect regulation of mRNAs [33].

Several studies have shown the miRNAs importance in the modulation of different biological processes [8, 34, 35]. New miRNAs are being discovered and their sequences deposited in the database for miRNAs, also known as miRBase [36]. So far, over 30 thousand sequences of mature miRNAs in more than 206 different species have been cataloged. The understanding of regulatory functions of miRNA and other small RNAs on the expression of target genes for lipogenesis and adipogenesis is important to identify targets molecules of fat deposition. These analyses can also provide base knowledge to further research to comprehend genetic modifications that lead to important metabolic diseases.

Although investigations studying the influence of miRNAs on energy metabolism are recent [37], there has been a rapid growth in the number of miRNAs identified that are involved in the regulation of genes and signaling molecules responsible for the maintenance of lipid homeostasis [8, 38]. Many studies have been published in this area, showing the importance of miRNAs as biomarkers, aiming to develop strategies to manipulate traits such intramuscular fat content, which affect the quality of meat and animal productivity [39, 40] and even in research on obesity and diabetes in humans [41].

The miR-122 was the first miRNA identified to regulate lipid metabolism, being initially described as affecting the level of hepatic cholesterol [42, 43]. Some recent studies have shown that anti-miR-122 therapy, i.e. molecules complementary to miRNAs that prevent its action, resulted in a 25-30% reduction in circulating cholesterol levels [44-46]. Its mechanism of action is due to direct or indirect downregulation of innumerable genes expressed in the liver that are involved in cholesterol biosynthesis, such as 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), 3-hydroxy-3-methylglutaryl-coenzyme synthase 1 (HMGCS1), 7-dehydrocholesterol reductase (DHCR7) and microsomal TG transfer protein (MTTP).

Another miRNA that has been extensively studied for modulating the expression of genes involved in lipid metabolism is miR-33 [47]. The family of this miRNA includes two members, miR-33a and miR-33b,

which are located in intronic regions within the genes of the transcription factors SREBP-2 and SREBP-1, respectively [48]. This transcription factor family, SREBP, regulates the expression of many genes involved in biosynthesis and uptake of cholesterol and fatty acids, as well as in the production of phospholipids and TAGs. SREBP-1 mainly regulates genes such as fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase (SCD), while SREBP-2 preferentially controls genes important for cholesterol metabolism including HMGCR and low-density lipoprotein receptor (LDLR) [8, 49]. Both miRNAs are transcribed along with their host genes, regulating physiological processes similar to them, and the mature sequence of these two miRNAs differ in only two nucleotides and have several common target genes, thus they have similar regulation effect for the organism [8, 38].

The miRNAs miR-33a and b have an important role in the post-transcriptional regulation of the expression of the ATP binding cassette transporter gene (ABCA1), which leads to the higher efflux of free cholesterol from within the cell to apolipoprotein A-1 (ApoA1), [50]. It is also important in high density lipoprotein (HDL) synthesis. Studies that have made the in vivo silencing of miR-33 have confirmed its action in the metabolism of cholesterol, because the results obtained with the technique showed increased HDL plasma levels and reduced cholesterol efflux, due to the influence of miR-33 in many genes involved in this process [51, 52]. Another target gene of miR-33 is its host gene SREBP-1, leading to a self-feedback regulation for proper lipid homeostasis, especially in relation to cholesterol biosynthesis in the body [53].

The mechanism of regulation of miRNA expression is dynamic and specific, and may vary from one tissue to another and also depending on the physiological conditions of the organism [54]. It is possible that the activity of the miRNAs is controlled by external stimulus, triggered by response to physiological changes. Thus, miRNAs are likely to serve as potential biomarkers for metabolic diseases, responses to therapeutic treatments, or as targets for gene therapies related to lipid metabolism in diseases such as diabetes and obesity [38, 55].

Although RNA-seq analysis has improved genomic studies and generates a large list of expressed genes in specific tissues, the biological interpretation of this data is still a challenge. The integration of genomic information, proteins, metabolites and cellular processes becomes very complex and it is still difficult to connect the molecular and cellular areas to understand the metabolism as a whole. Thus, the systems biology study, that is the integrative analysis of biological interaction networks, is so important to take advantage of the information provided by all genes and regulatory molecules, such miRNAs, found in RNA-seq and have become an interesting strategy for the development of therapies using miRNAs as therapeutic targets [56].

1.3 Hypothesis

Gene and microRNA expression, and co-expression networks can influence intramuscular fat deposition, as well as the metabolic pathways they participate.

1.4 Objectives

To identify the importance of miRNAs-mRNAs networks in the regulation of gene expression and how it affects the content of intramuscular fat in Nelore cattle by analysis of a groups of animals with extreme genomic estimated breeding values (GEBV).

1.4.1 Specific objectives

1) Analyze the differential expression of miRNAs between the two different group of animals;
2) Identify potential target genes of those differentially expressed miRNAs in muscle;
3) Identify gene networks from a list of miRNAs target genes;
4) Identify miRNAs-mRNAs co-expression networks;
5) Identify candidate regulators genes involved in intramuscular fat deposition process;
6) Identify biological processes and pathways associated with intramuscular fat deposition.

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2 INTEGRATIVE ANALYSIS OF MICRORNAS AND MRNAS REVEALED REGULATION OF INTRAMUSCULAR FAT DEPOSITION IN NELORE CATTLE

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Abstract

The amount of intramuscular fat can influence the sensory characteristics and nutritional value of beef, thus the selection of animals with adequate fat content is important for consumer. Intramuscular fat is a complex trait that is difficult to measure and there is growing knowledge about the genes and pathways that control the biological processes involved in fat deposition in muscle. MicroRNAs (miRNAs) belong to a well conserved class of non-coding small RNAs that modulate gene expression across a range of biological functions in animal development and physiology. The aim of this study was to identify differentially expressed (DE) miRNAs, regulatory candidate genes and co-expression networks related to intramuscular fat (IMF) content. To achieve this we used mRNA and miRNA expression data from the *Longissimus dorsi* muscle of 30 Nelore steers with extreme genomic estimated breeding values (GEBV) for this trait. Differential miRNA expression analysis between animals with extreme GEBV values for IMF identified six DE miRNAs. Functional annotation of the target genes for these microRNAs indicated that PPARs signaling pathway maybe is involved with IMF deposition. Regulatory candidate genes such as SDHAF4, FBXO17, ALDOA and PKM were identified by partial correlation with information theory (PCIT), phenotypic impact factor (PIF) and regulatory impact factor (RIF) approaches from integrated miRNA-mRNA expression data. Two DE miRNAs, bta-miR-143 and bta-miR-146b, which were upregulated in the Low IMF group, were correlated with regulatory candidate genes, which were functionally enriched for GO terms for fatty acids oxidation. Co-expression patterns obtained by weighted correlation network analysis (WGCNA), which showed possible interaction and regulation between mRNAs and miRNAs, identified several modules related to immune system, protein metabolism, energy metabolism and glucose catabolism. In this study, several genes and miRNAs were identified as possible regulators of IMF by analyzing DE miRNAs and using different strategies for identification of miRNAs-mRNAs co-expression networks. This study contributes to the understanding of regulatory mechanisms of gene signaling networks involved in fat deposition process. Glucose metabolism and inflammation process were the main pathways found in integrative mRNAs-miRNAs analysis and showed to influence intramuscular fat content in beef cattle.

Keywords: lipids, RNA-Seq, *Bos indicus*, microRNAs, co-expression networks.

2.1 Introduction

The amount of intramuscular fat (IMF) is an important characteristic associated with juiciness and taste of beef [1]. Overall meat quality can be impacted by many factors such as nutritional program, genetic, environment, age and sex. For example, zebu animals (*Bos taurus indicus*) slaughtered at an earlier age present a more homogeneous distribution of subcutaneous fat but lower amount of intramuscular fat in relation to *Bos taurus taurus* animals [2, 3]. This beef quality characteristic, i.e. low IMF, is also an important economic trait because for some

markets in Continental Europe, lower amounts of fat in the carcass are desirable, but for others, like some countries in Asia and the UK, this feature lowers the market value of Brazilian beef. Despite the prominent position of the beef cattle industry in the economy, Brazil still exports meat without added value to international market. Thus, the control of beef quality, and their sensory characteristics (tenderness, flavor, juiciness and color) are very important.

MicroRNAs (miRNAs) are endogenous non-coding (ncRNA) ribonucleic acids (RNAs) that are approximately twenty-two nucleotides in length [4]. These molecules modulate the expression of genes at the post-transcriptional level by blocking the translation of target mRNAs [4]. The first miRNA was discovered in Caenorhabditis elegans and was called lin-4 as it was involved in the suppression of a gene that encodes the lin-4 protein [5]. Lin-4 acts in larval development of this nematode species.

MiRNAs play an important role in post-transcriptional gene regulation in many tissues and are associated with the control of several important biological processes related to lipid metabolism [6]. Understanding the regulatory functions of miRNA and other small RNAs on the expression of target genes impacting lipogenesis and adipogenesis is important to identify target molecules with potential impact on fat deposition. Several studies have been published, which demonstrate the importance of miRNAs as potential biomarkers for variations in IMF [7-9]. Once identified biomarkers could be used to improve the quality of meat and animal productivity [7, 9] and potentially provide priors for the research in human diseases such as obesity and diabetes [10, 11]. Identification of new regulatory roles for miRNA in lipid metabolism would be important in understanding potential mechanisms involved in metabolic diseases.

Although RNA-seq analyses can be helpful for genomic studies and can generate lists of expressed genes in specific tissues to ultimately detect differentially expressed (DE) genes, the biological interpretation of this data is still a challenge. Network approaches that integrate data have proven useful in the identification of complex transcriptional regulation. For example, hub genes, which are highly correlated with a large number of genes, have been shown to have key regulatory roles in gene expression networks [12-14]. Thus, co-expression analysis may be more sensitive at detecting biologically interesting pathways than analysis of DE genes [15]. Several network approaches are available for this purpose, such as the Weighted Gene Co-expression Network Analysis (WGCNA) method and the Partial Correlation with Information Theory (PCIT) methods. The WGCNA method identifies gene correlation networks, i.e. gene clusters of biological significance, from expression profiling data [16]. The PCIT method identifies differences in pairs of correlated gene expression levels to measure a gene's differential connectivity across levels of a phenotype [17]. Both the PCIT and WGCNA approaches have enabled a better understanding of the co-regulation of mRNAs and miRNAs for different phenotypes [15, 18-21] to better comprehend the biological mechanisms and regulatory processes in lipid metabolism.

The aim of this study was to identify candidate regulatory genes and pathways that are regulated by miRNAs and understand the importance of integrative co-expression networks of mRNAs-miRNAs for fat deposition in cattle from a systems biology perspective.

2.2 Results

2.2.1 Phenotypic and sequencing data

The genetic variance, residual variance and heritability for IMF obtained from this population were 0.196, 0.490 and 0.29±0.16, respectively, as previously published [22]. The animals were ranked using GEBV for IMF values and fifteen animals with high IMF GEBV (H) and fifteen with low IMF GEBV (L) were selected for miRNA-Seq analysis (Table 1). This strategy, to select animals with extreme GEBV, was performed because the correlation between the raw IMF values (% IMF) and GEBV was high (r = 0.76) [22] and the statistical T-test showed that the GEBV averages for groups were statistically different (p-value = 2.2e-16).

A total of 32 million (M) reads were obtained from sequencing on Illumina MiSeq equipment. The average number of total reads per sample was one million. The reads were filtered using the FASTX program (total number of filtered reads was 28 M) and mapped by miRDeep2. The total number of mapped reads was 24 M, for an average of 84 % reads mapped (Table 1).

Table 1. Phenotypic data for intramuscular fat percentage (IMF), genomic estimated breeding values (GEBV) and number of normalized mapped miRNA reads of all animals.

Animal	IMF (%)	GEBV	Mapped Reads
High[1]	4.42	0.44	676,705.83
High[2]	4.12	0.51	722,149.21
High[3]	4.35	0.57	856,445.98
High[4]	5.02	0.47	564,721.48
High[5]	4.74	0.81	1,477,652.40
High[6]	3.99	0.51	382,812.08
High[7]	4.17	0.66	1,372,859.97
High[8]	4.95	0.59	714,291.96
High[9]	3.97	0.57	637,211.41
High[10]	4.38	0.71	628,643.15
High[11]	5.27	0.85	803,295.42
High[12]	4.35	0.61	675,159.48
High[13]	3.75	0.42	610,860.91
High[14]	2.99	0.36	327,429.32
High[15]	4.13	0.81	578,590.62
Low[1]	2.06	-0.57	681,969.11
Low[2]	1.32	-0.77	825,926.83
Low[3]	1.35	-0.36	654,790.36
Low[4]	1.7	-0.31	510,809.28
Low[5]	1.44	-0.51	661,870.55
Low[6]	1.04	-0.33	675,033.41
Low[7]	1.58	-0.5	711,330.33
Low[8]	1.39	-0.52	421,474.63
Low[9]	1.94	-0.29	727,950.69
Low[10]	1.86	-0.24	980,827.28
Low[11]	1.38	-0.43	754,990.11
Low[12]	1.6	-0.59	655,706.88
Low[13]	1.62	-0.57	862,654.84
Low[14]	0.65	-0.22	821,821.32
Low[15]	1.69	-0.27	1,398,620.62
Mean High	4.306	0.592	735,255.28
Mean Low	1.508	-0.432	756,385.08

2.2.2 Differentially expressed microRNAs and target genes identification

Twenty-six novel and 463 known miRNAs were identified using miRDeep2 analysis. Among all miRNAs identified, six of them were DE with a False Discovery Rate (FDR) of 10% (Table 2). Negative values of fold change indicate lower miRNA expression in animals with low IMF content and positive values indicate higher miRNA expression for this group. These six miRNAs targeted 2,250 genes expressed in skeletal muscle based on IPA analysis. Of note, because bta-let-7f and bta-let-7a-5p belong to the same family of miRNAs, they have the same seed sequence, and therefore target the same genes (Table 2).

Table 2. List of differentially expressed miRNAs between Low and High groups based on genomic estimated breeding values (GEBV) for intramuscular fat identified by miRDeep2 and the number of target genes obtained by IPA® for each miRNA.

miRNA	FDR[1]	Fold Change[2]	Low[3]	High[4]	Target Genes[4]
bta-let-7f	0.04	-1.671	2617.43	3767.18	1236
bta-let-7a-5p	0.08	-1.456	1526.04	1908.20	1236
bta-miR-146b	0.08	1.557	423.46	301.78	544
bta-miR-100	0.09	1.715	1968.02	840.72	176
bta-miR-143	0.09	1.309	32275.17	27539.78	648
bta-miR-423-5p	0.09	-1.600	311.91	488.05	294

[1] False discovery rate adjusted p-values by Benjamini-Hochberg methodology

[2] Log2 Fold Change from low to high groups

[3] Mean normalized counts from low and high groups

[4] Target genes identified by IPA

2.2.3 Enrichment analysis of target genes

Functional enrichment analyses of target genes by IPA showed networks and canonical pathways related to fatty acid metabolism (Table 3; Figs. 1 and 2; Annex A: Figs. 14 and 15; Annex B: Figs 16 and 17). The most relevant gene network was "lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism" that involved genes such as PPARGC1A, MYCN, ESR2 and ARL4D, that are targets of downregulated miRNAs and MED1, SMAD4, NEDD4 and MBOAT2, that are targets of upregulated miRNAs in the L group (Fig. 1).

Table 3. List of the top gene networks and signaling pathways related with lipid metabolism identified by IPA®.

Gene Networks	Target genes	Signaling Pathways	Target genes	P-value[1]
Drug Metabolism, Lipid Metabolism, Molecular Transport	32	PPAR Signaling	33	1.00E-08
Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism	31	PPARa-RXR Activation	44	3.00E-06
Gene Expression, Cell Cycle, Cancer	32	Adipogenesis	27	0.003

[1] Nominal p-value, not adjusted

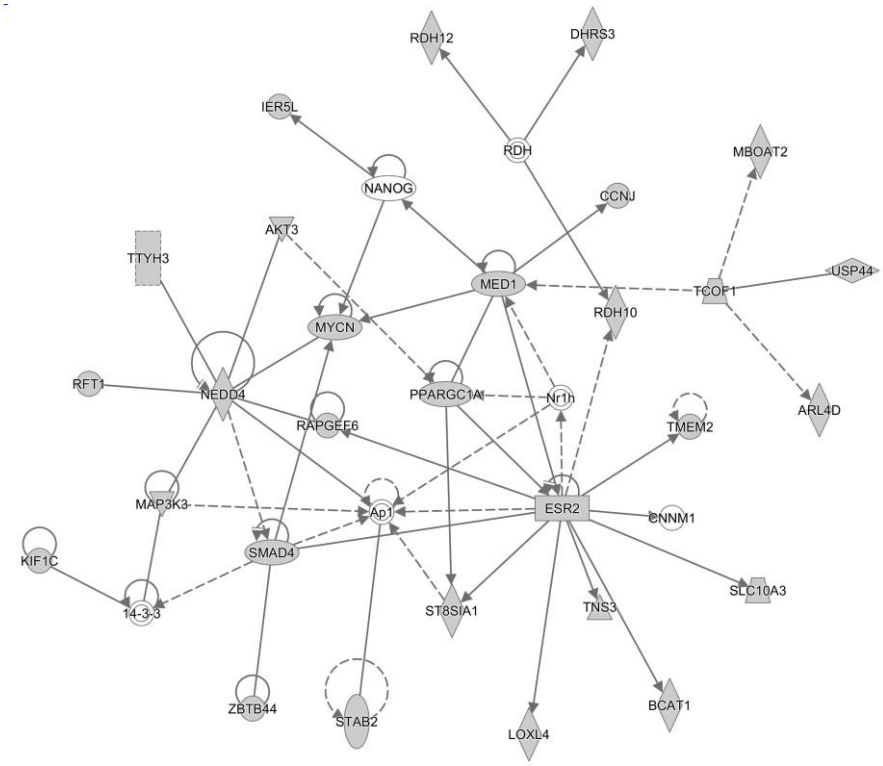

Figure 1. Gene network "lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism" identified from miRNAs target gene list by IPA. Grey shapes represent target genes and the white shapes are other genes of the network that are not target genes. Solid lines mean direct interaction and dashed lines an indirect interaction between genes.

Target genes enriched for PPAR-RXR signaling pathways (i.e. lipogenesis promoting) were negatively regulated by miRNAs which were upregulated in L group. Target genes enriched for fatty acid oxidation were targets of downregulated miRNAs. Some important genes for lipid metabolism present in this pathway included: PPARα, PKA and ADIPOR2. These genes are targets of the downregulated miRNAs bta-let-7 and bta-miR-423 (i.e. downregulated in the L group). On the other hand, STAT5b and GPDH are targets of upregulated miRNAs (bta-miR-100 and bta-miR-143) in L group (Fig. 2).

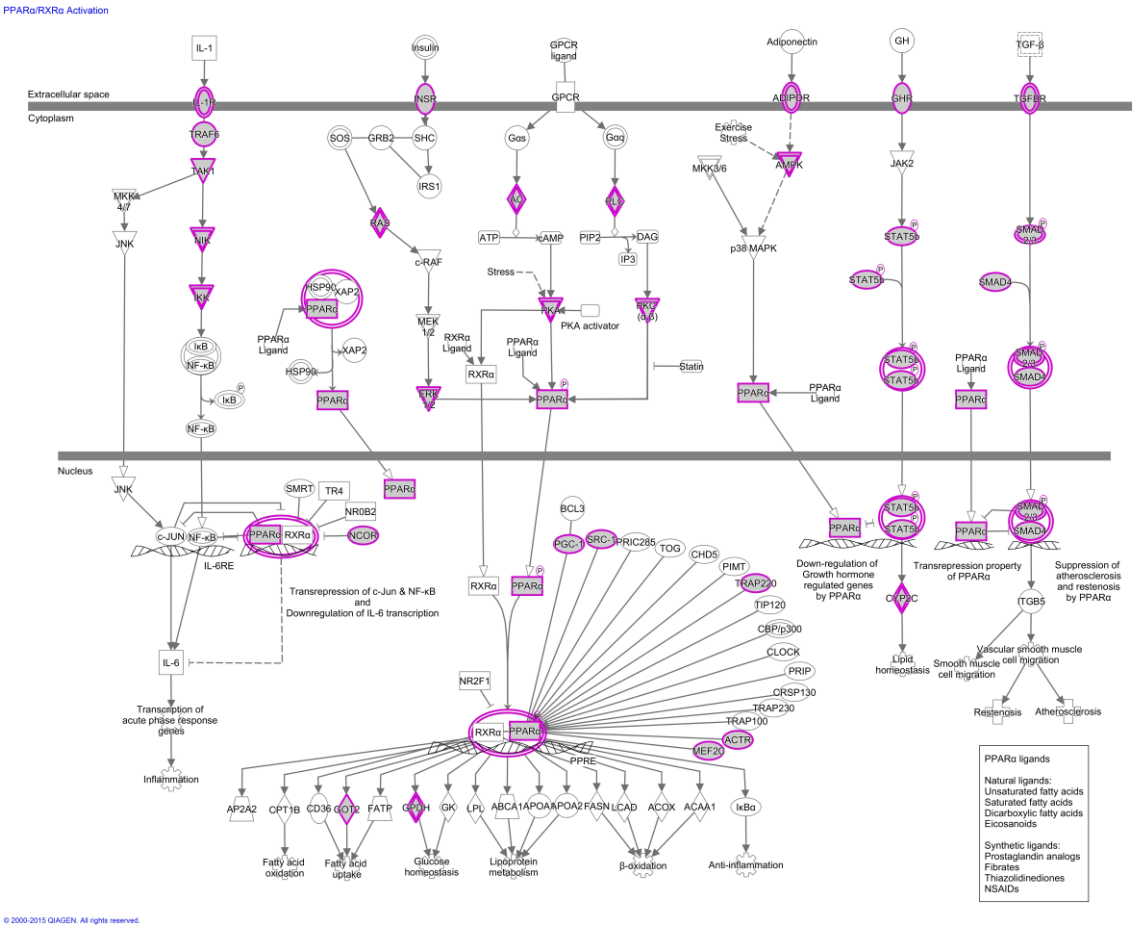

Figure 2. PPARα-RXR signaling pathway overrepresented in miRNA target genes list by IPA. The shapes highlighted in purple represent the miRNAs target genes and the white shapes represent the other genes of the pathway that are not target genes.

2.2.4 Co-expression analysis: PCIT – Differential Hubbing

After data filtering by expression in H and L IMF groups, a list with 383 miRNAs and 14,650 genes expressed in bovine skeletal muscle were used for PCIT analysis that allowed the identification of ten positive and negative differentially hubbed (DH) genes and miRNAs (Table 4).

Table 4. List of the top ten positive and negative Differentially Hubbed (DH) genes, when comparing High and Low groups of GEBV for IMF.

ENSEMBL Gene ID	Gene Symbol	DH
Top Positive Differentially Hubbed genes		
ENSBTAG00000009084	*ATG3*	1849
ENSBTAG00000005688	*MRPS2*	1793
ENSBTAG00000008664	*EIF2B2*	1785
ENSBTAG00000012113	*HCCS*	1781
ENSBTAG00000005196	*TYW3*	1755
ENSBTAG00000001022	*AMDHD2*	1750
ENSBTAG00000010339	*ABHD11*	1736
ENSBTAG00000017941	*NSUN5*	1735
ENSBTAG00000003066	*NSA2*	1731
ENSBTAG00000001783	*FBXO17*	1730
Top Negative Differentially Hubbed genes		
ENSBTAG00000027049	*SDHAF4*	-851
ENSBTAG00000010952	*C2CD4B*	-850
ENSBTAG00000005275	*PKIG*	-837
ENSBTAG00000009876	*C4BPA*	-835
ENSBTAG00000011184	*FTH1*	-828
ENSBTAG00000008895	*BPGM*	-819
-	bta-miR-24-3p	-811
-	bta-miR-1291	-810
ENSBTAG00000031778	*HIST1H2BD*	-799
ENSBTAG00000038275	*CYP27C1*	-795

The genes with a significant correlation with DH genes were used to construct co-expression networks and identify enriched GO terms. The GO terms enriched among all genes correlated to the top ten negative DH genes were most related to glucose metabolism (GO ID: 6006, GO ID: 6007, GO ID: 6096) (Fig. 3) and for the top ten positive DH genes the GO terms were related to protein and mRNA metabolism (GO ID: 6364, GO ID: 6350, GO ID: 30163, GO ID: 30162, GO ID: 51603) (Fig. 4).

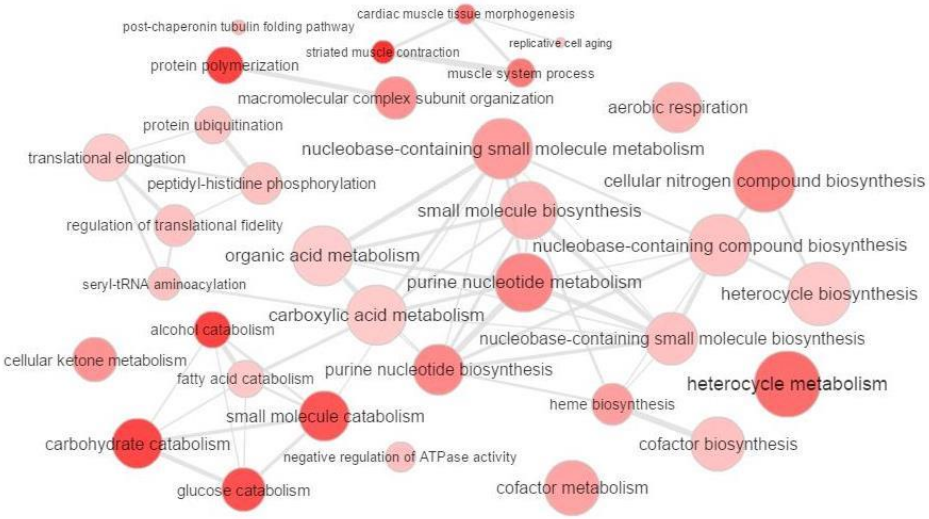

Figure 3. GO terms enriched from genes correlated with negatively differentially hubbed genes. Bubble color indicates the user-provided p-value; bubble size indicates the frequency of the GO term in the underlying GOA database. Highly similar GO terms are linked by edges in the graph, where the line width indicates the degree of similarity.

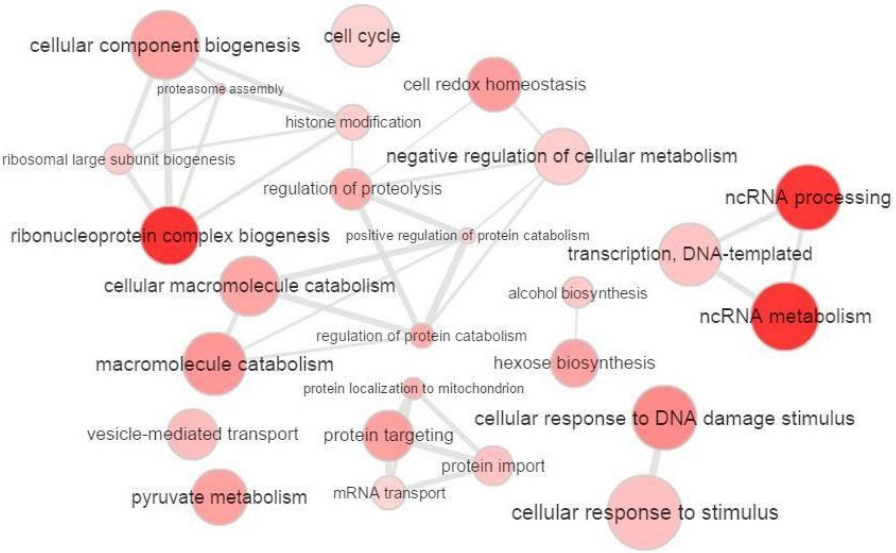

Figure 4. GO terms enriched from genes correlated with positively differentially hubbed genes. Bubble color indicates the user-provided p-value; bubble size indicates the frequency of the GO term in the underlying GOA database. Highly similar GO terms are linked by edges in the graph, where the line width indicates the degree of similarity.

The most important DH genes potentially involved in the regulation of lipid metabolism and protein metabolism are shown in Table 5. The negatively DH genes bta-miR-24-3p and SDHAF4 are important for differentiation of adipocytes [23] and the assembly of succinate dehydrogenase, respectively. The positively DH gene FBXO17 is a component of the Skp, Cullin, F-box (SCF) complex, which mediates ubiquitination of proteins that leads to degradation and can bind to glycoprotein substrates [24]. Another positively DH gene is

EIF2B2, an eukaryotic initiation factor that is required in the initiation of translation and protein synthesis [25]. The co-expression networks of top DH genes were visualized by BioLayout (Fig. 5).

Table 5. List of the top two differentially hubbed (DH) genes and the GO terms associated with them. The negative DH genes have higher number of connections in Low GEBV group and positive DH genes in High GEBV group.

ENSEMBL Gene ID	Gene Symbol	DH	GO terms of genes correlated
Top Negative Differentially Hubbed genes			
ENSBTAG00000027049	*SDHAF4*	-851	GO ID 44275:cellular carbohydrate catabolic process GO ID 44282:small molecule catabolic process GO ID 16052:carbohydrate catabolic process
	bta-miR-24-3p	-811	GO ID 44275:cellular carbohydrate catabolic process GO ID 6096:glycolysis GO ID 6936:muscle contraction
Top Positive Differentially Hubbed genes			
ENSBTAG00000008664	*EIF2B2*	1785	GO ID 6090:pyruvate metabolic process GO ID 30162:regulation of proteolysis GO ID 6364:rRNA processing
ENSBTAG00000001783	*FBXO17*	1730	GO ID 30162:regulation of proteolysis GO ID 6364:rRNA processing GO ID 70585:protein localization in mitochondrion

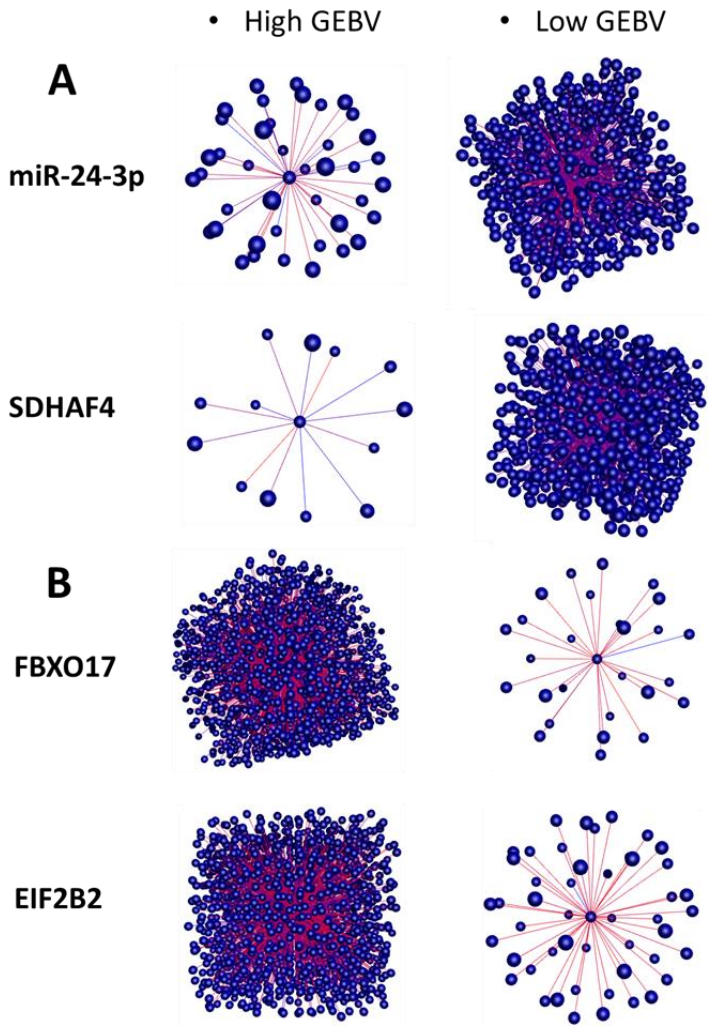

Figure 5. Negative (A) and positive (B) differentially hubbed genes associated with lipid metabolism between groups High GEBV and Low GEBV. The blue edges mean negative correlations between hub gene and the other connected genes and the red edges mean positive correlations.

2.2.5 Co-expression analysis: PIF and RIF

The PIF and RIF analyses were used to identify putative regulatory genes that may explain differences in phenotype between groups of animals, based on differences in gene expression. The most relevant genes for fatty acid metabolism found in the RIF and PIF analyses and the GO terms associated with them are shown in Table 6. The genes with positive values for RIF 1 and 2 were PYGM, ENO3, ATP2A1, GAPDH and ALDOA, which were principally related to glucose metabolism and energy metabolism. Those with negative values of RIF were mostly miRNAs. Specifically listed in the RIF 2 as one of the genes with most extreme negative value is the miRNA bta-miR-143, which was identified as DE in this study (Table 1). The bta-miR-26b, which plays a role in cholesterol efflux [26], had an extreme negative RIF2 value. The PIF analysis identified ALDOA as a putative regulatory gene for the difference in fat content between H and L groups. The enrichment of GO terms was similar with those found in DH analysis.

Table 6. List of the genes with the most extreme Phenotypic Impact Factor (PIF) and Regulatory Impact Factor (RIF) 1 and 2 values and the GO terms associated with them.

ENSEMBL Gene ID	Gene Symbol	Score	GO terms of genes correlated/targets
		Top PIF	
ENSBTAG00000012927	*ALDOA*	4.895E+10	GO ID 30163:protein catabolic process GO ID 6006:glucose metabolic process GO ID 6091:generation of precursor metabolites and energy
		Top Positive RIF1 and 2	
ENSBTAG00000012927	*ALDOA*	105.5434	GO ID 6006:glucose metabolic process
ENSBTAG00000005534	*ENO3*	4.121744	GO ID 6007:glucose catabolic process
ENSBTAG00000001032	*PYGM*	6.417047	GO ID 16052:carbohydrate catabolic process
ENSBTAG00000014731	*GAPDH*	4.403429	GO ID 22900:electron transport chain
ENSBTAG00000006541	*ATP2A1*	5.891178	GO ID 6006:glucose metabolic process
		Top Negative RIF2	
ENSBTAG00000001601	*PKM*	-0.5742	GO ID 6006:glucose metabolic process
ENSBTAG00000030114	bta-miR-143	-0.74594	GO ID 6538:glutamate catabolic process
ENSBTAG00000029850	bta-miR-26b	-0.84051	GO ID 6793:phosphorus metabolic process

2.2.6 Co-expression analysis: WGCNA - miRNAs correlated with mRNA modules

The WGCNA methodology was applied in two different manners, first to integrate mRNAs and miRNAs by analyzing those modules with a negative correlation between them and second by identifying modules that are important to phenotypic variation by correlating all modules identified in WGCNA to IMF content. The dendrograms resulting from clustering of genes and miRNAs, made in separately analysis, are presented in Figures 6 and 7, respectively. The lowest soft threshold power (β) with scale free fitting index of 0.9 was applied to calculate the adjacency matrix of mRNAs and miRNAs from H and L groups (Figs. 8 and 9). The β's used to construct the mRNA modules from the L and H IMF group's expression data were 12 and 8, respectively. While the β's used to construct the miRNA modules were 9 for the L and 4 for the H IMF group. A total of 27 mRNA modules in H and 44 in the L group were identified, and were 14 miRNA modules in H and 22 in L. The grey module contained all genes not included in a correlated module.

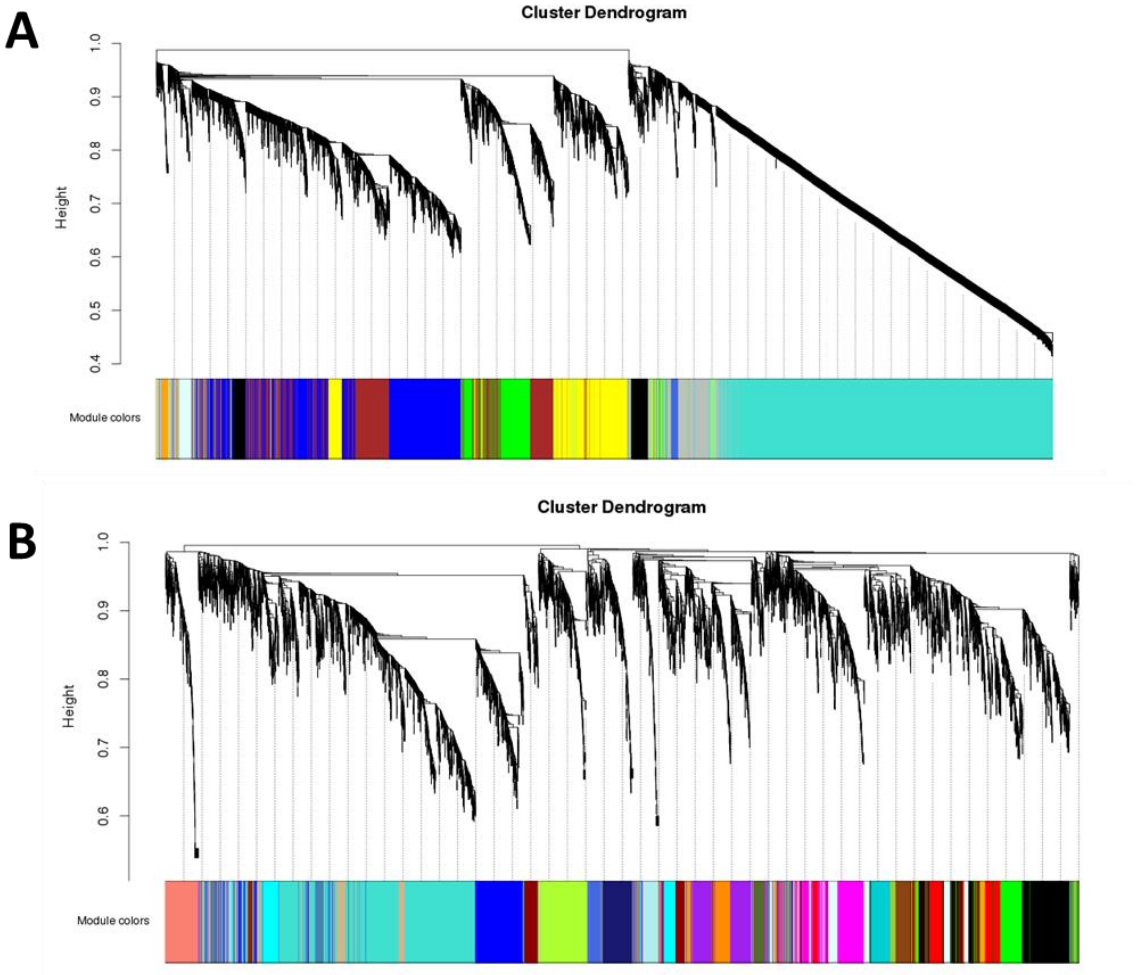

Figure 6. The color-band below the dendrogram denotes the mRNAs modules, which are defined as branches in the dendrogram. A: High IMF GEBV group network. B: Low IMF GEBV group network.

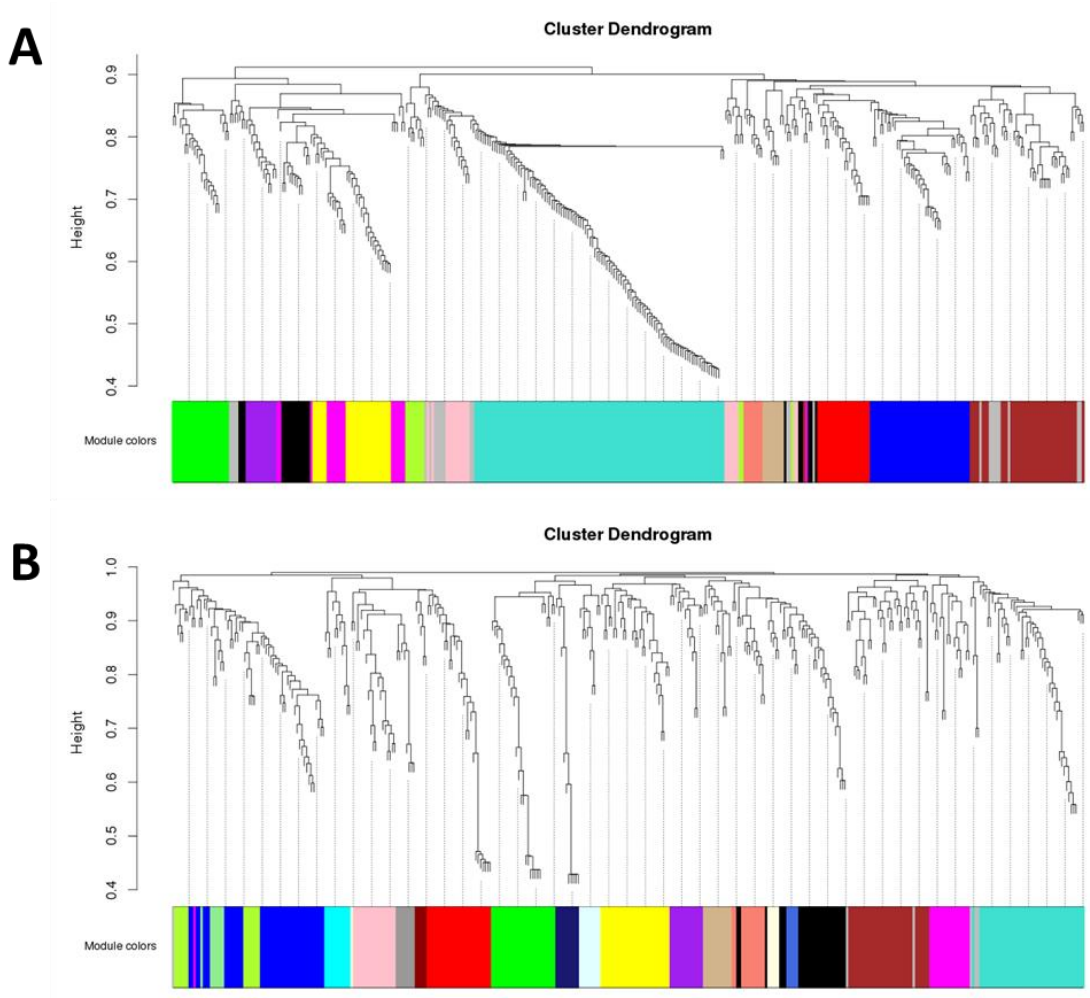

Figure 7. The color-band below the dendrogram denotes the miRNAs modules, which are defined as branches in the dendrogram. A: High IMF GEBV group network. B: Low IMF group GEBV network.

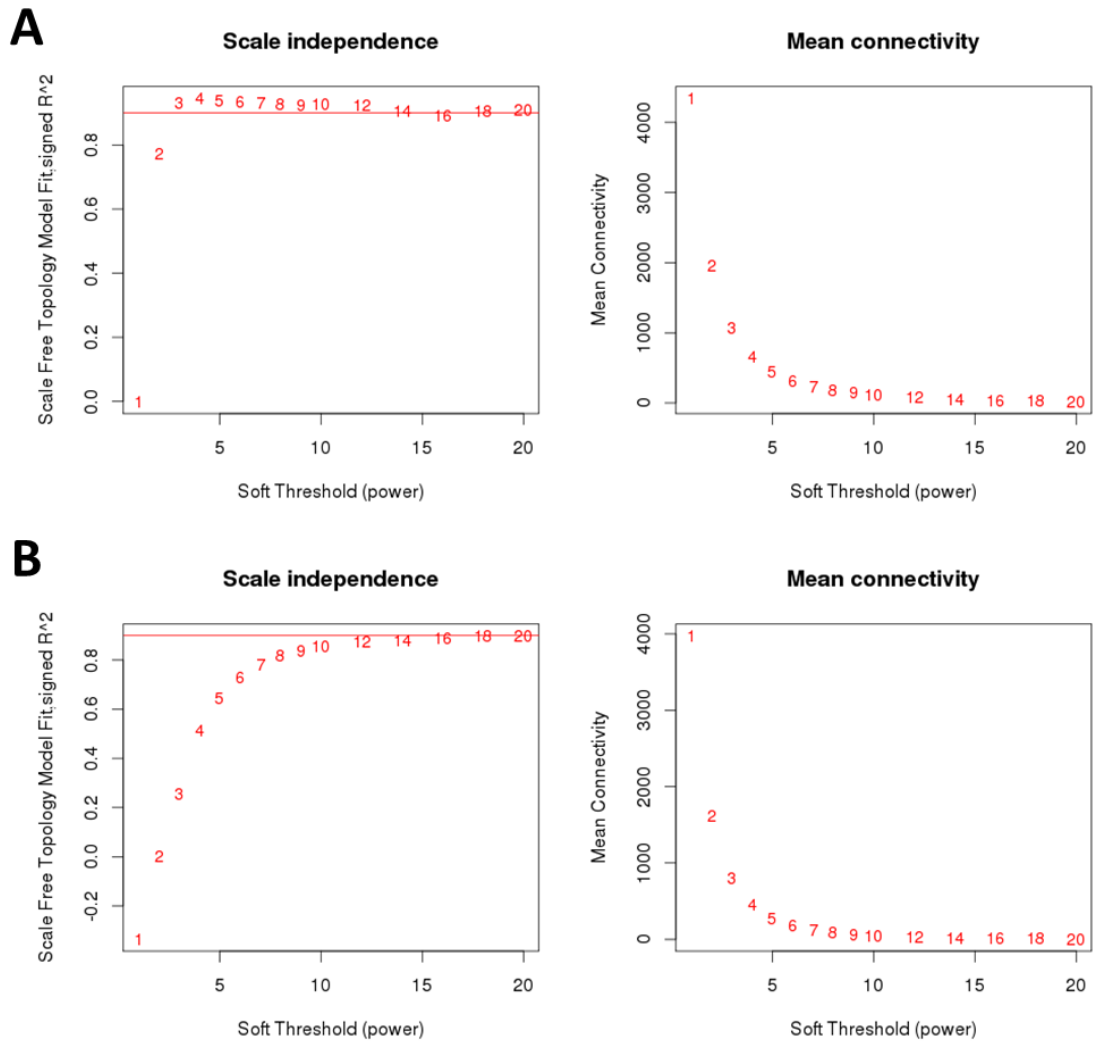

Figure 8. Scale free topology model and Mean connectivity of mRNAs network on the basis of power β value. (A) H group. (B) L group.

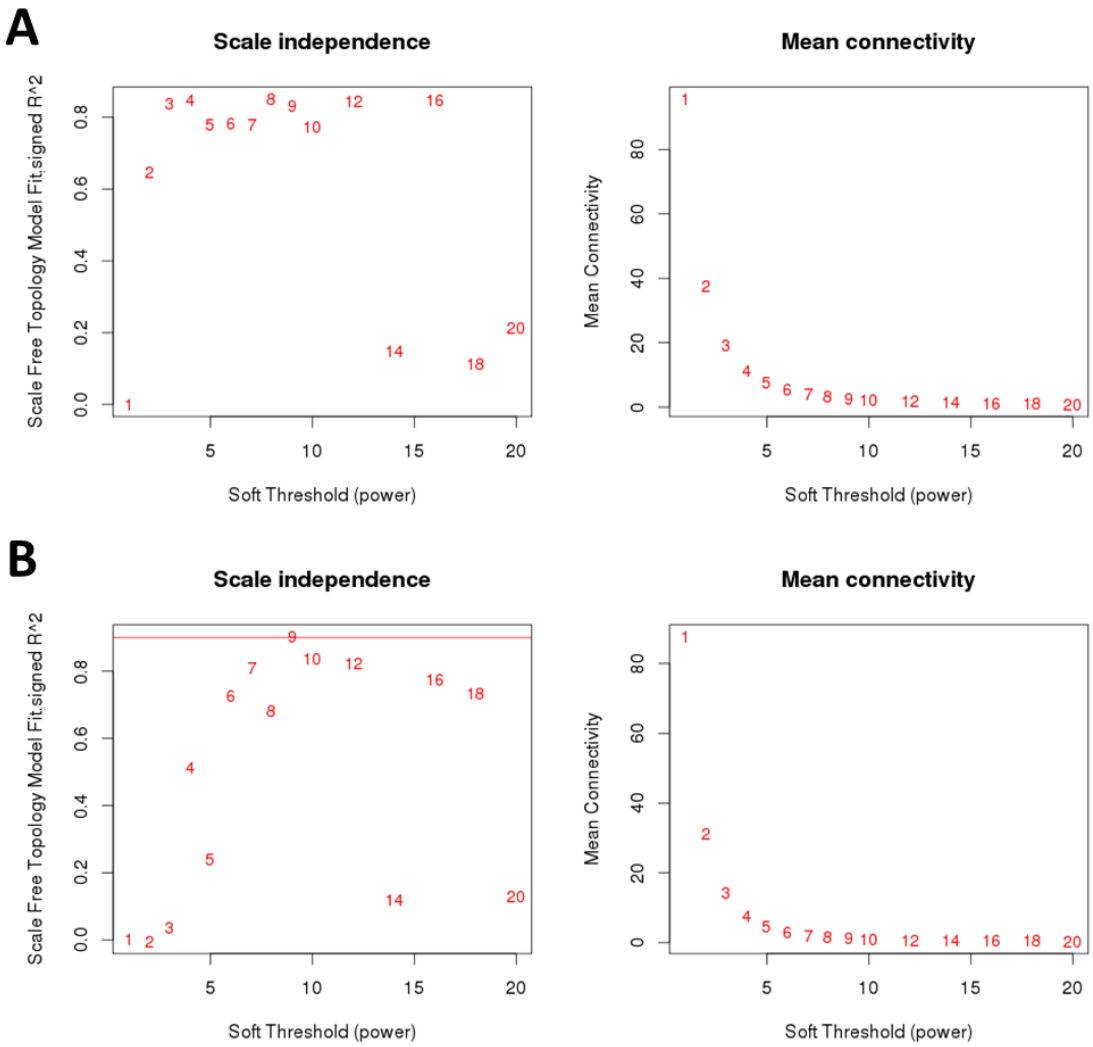

Figure 9. Scale free topology model and Mean connectivity of miRNAs network on the basis of power β value. (A) H group. (B) L group.

After correlating all miRNA and mRNA modules, those modules that were negatively correlated with one another were investigated further. Among all correlated modules, three miRNA modules were negatively correlated with five mRNAs modules in the H group, while six miRNA modules were negatively correlated with seven mRNA modules in the L group (p-value > 0.05; Table 7). The genes that composed each mRNA module were significantly over enriched for GO terms most related to lipid metabolism (adj. p-value <0.1). These lipid metabolism GO terms enriched from mRNA modules were then used to construct mRNA-miRNA co-expressed networks for both groups H and L (Figs. 10 and 11). The miRNAs modules were enriched for GO terms based on the hub miRNA target genes.

Table 7. List of miRNAs modules negatively correlated with mRNAs modules.

Group	miRNAs Modules	mRNA Modules	Correlation	p-value	#miRNAs	#mRNAs	#targets
H	black	cyan	-0.853	(0.0000518)	21	187	17
	green	pink	-0.625	(0.012)	24	295	64
	green	blue	-0.681	(0.005)	24	2004	707
	green	brown	-0.668	(0.006)	24	1542	615
	pink	lightgreen	-0.542	(0.036)	20	152	13
L	midnightblue	green	-0.637	(0.01)	10	743	360
	midnightblue	orange	-0.531	(0.041)	10	174	85
	midnightblue	yellowgreen	-0.589	(0.02)	10	73	33
	midnightblue	black	-0.66	(0.007)	10	704	264
	lightcyan	black	-0.577	(0.024)	9	704	294
	lightcyan	lightyellow	-0.545	(0.035)	9	256	53
	cyan	darkolivegreen	-0.554	(0.031)	11	106	33
	magenta	orangered4	-0.514	(0.049)	18	60	8
	salmon	darkolivegreen	-0.55	(0.033)	12	106	21
	salmon	green	-0.551	(0.033)	12	743	131
	tan	green	-0.529	(0.044)	12	743	294
	tan	orange	-0.576	(0.024)	12	174	62

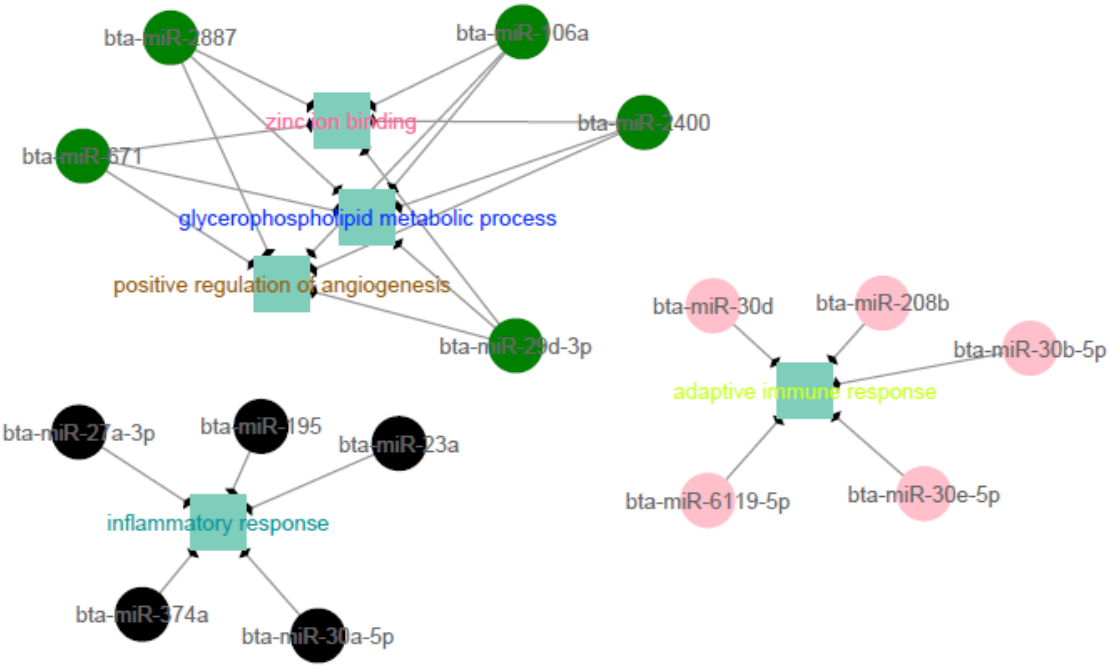

Figure 10. Co-expression networks showing the inverse correlation among miRNAs and biological processes in High IMF GEBV group. Colored circles represent hub miRNAs, with higher connectivity, inside each module and squares represent the GO terms associated with each mRNA module, represented by different letter color.

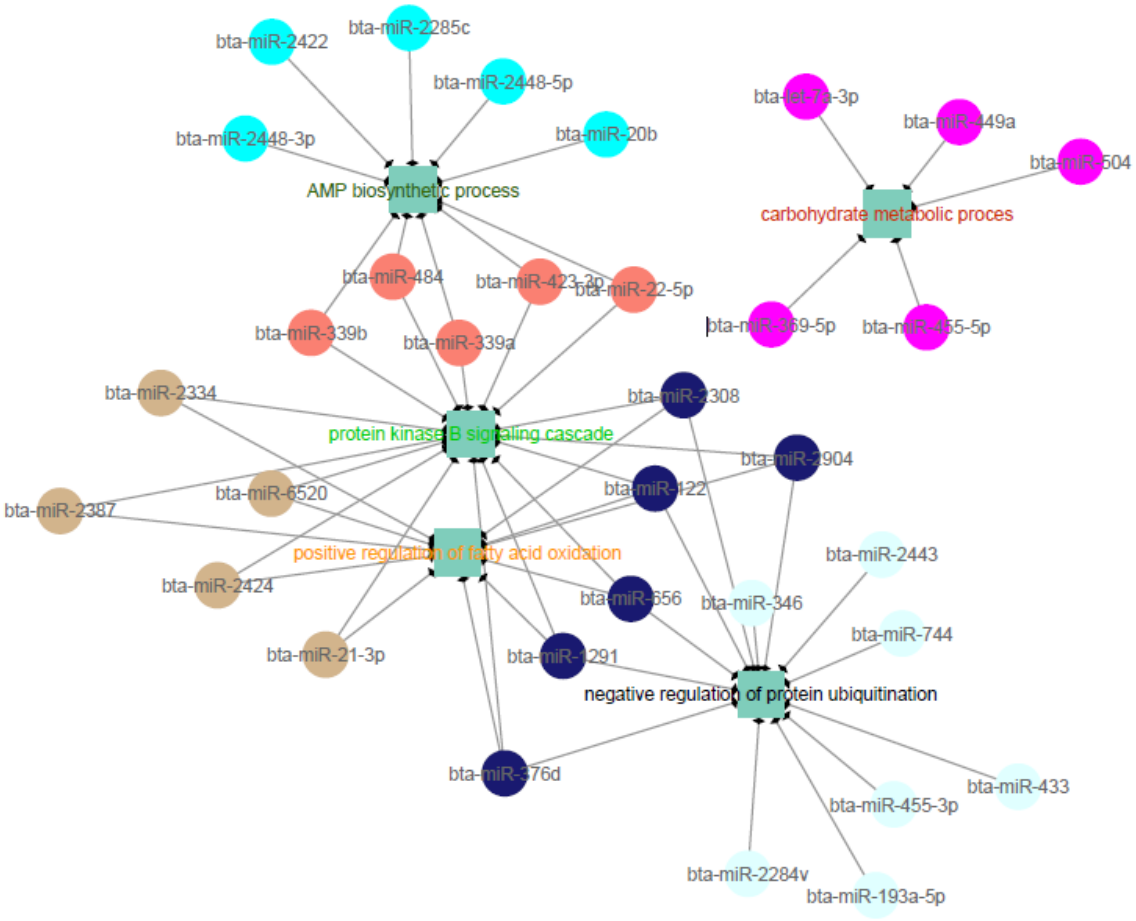

Figure 11. Co-expression networks show the inverse correlation among miRNAs and biological processes in Low IMF GEBV group. Colored circles represent hub miRNAs, with higher connectivity, inside each module and squares represent the GO terms associated with each mRNA module, represented by different letter color.

In the H group, the green miRNA module was negatively correlated with pink, blue and brown mRNA modules whose genes were enriched for GO terms related to zinc ion binding, angiogenesis and glycerophospholipid metabolism. The hub miRNAs in the black module were negatively correlated to genes enriched for inflammatory response (cyan mRNA module) and miRNAs in the pink module were negatively correlated to genes enriched for immune response (lightgreen mRNA module) (Fig. 10). The L group contained multiple miRNA modules negatively correlated with the same mRNA module (Table 7). Specifically, the miRNA modules midnightblue, tan and salmon were negatively correlated with mRNAs inside the green module, while the midnight blue and tan miRNA modules were negatively correlated with the orange mRNA module (Fig. 11). The orange mRNA module was enriched for positive regulation of fatty acid oxidation, while the green mRNA module was enriched for protein kinase B signaling cascade. The midnightblue miRNA module and the lightcyan miRNA module were negatively correlated with the black mRNA module, enriched for negative regulation of protein ubiquitination. The salmon miRNA module and the cyan miRNA module were negatively correlated with darkolivegreen mRNA module, enriched for AMP biosynthetic process. The magenta miRNA module was negatively correlated with orangered4 mRNA module, which was enriched for carbohydrate metabolic process. The yellowgreen mRNA module was not enriched for any biological processes (p-value > 0.10).

2.2.7 Co-expression analysis: WGCNA – miRNAs correlated with phenotype

Besides the integration of mRNA and miRNA data, the correlation of modules with the phenotype (high or low IMF content) was also performed (Figs. 12 and 13). Three mRNA modules in the H group and two in the L group, and three miRNA modules both the H and L group were correlated with the IMF phenotype (Table 8). In the H group, the black miRNA module and the cyan mRNA module were negatively correlated with each other (Table 7).

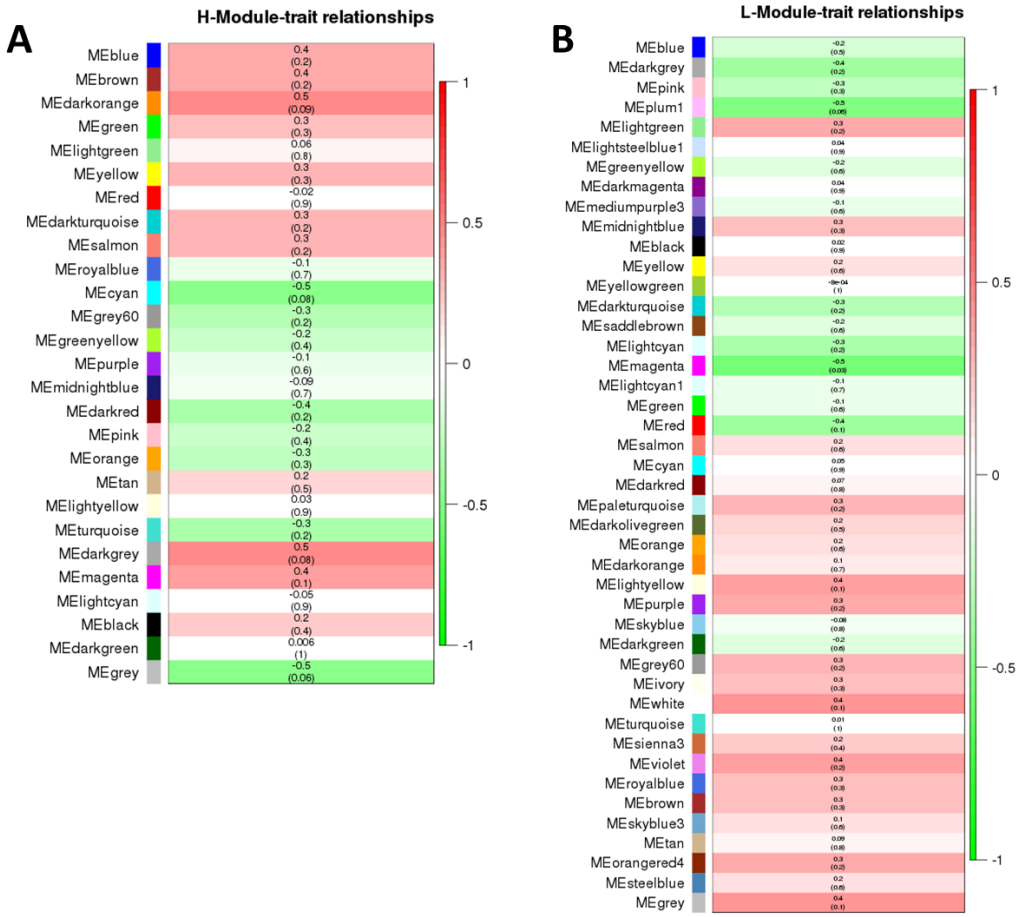

Figure 12. Correlation between the mRNA modules and IMF. A: modules identified in the High IMF GEBV (H) group. B: modules identified in the Low IMF GEBV (L) group. Modules with intense red color have a higher correlation (close to +1) and those with intense green color have a more negative correlation (close to -1).

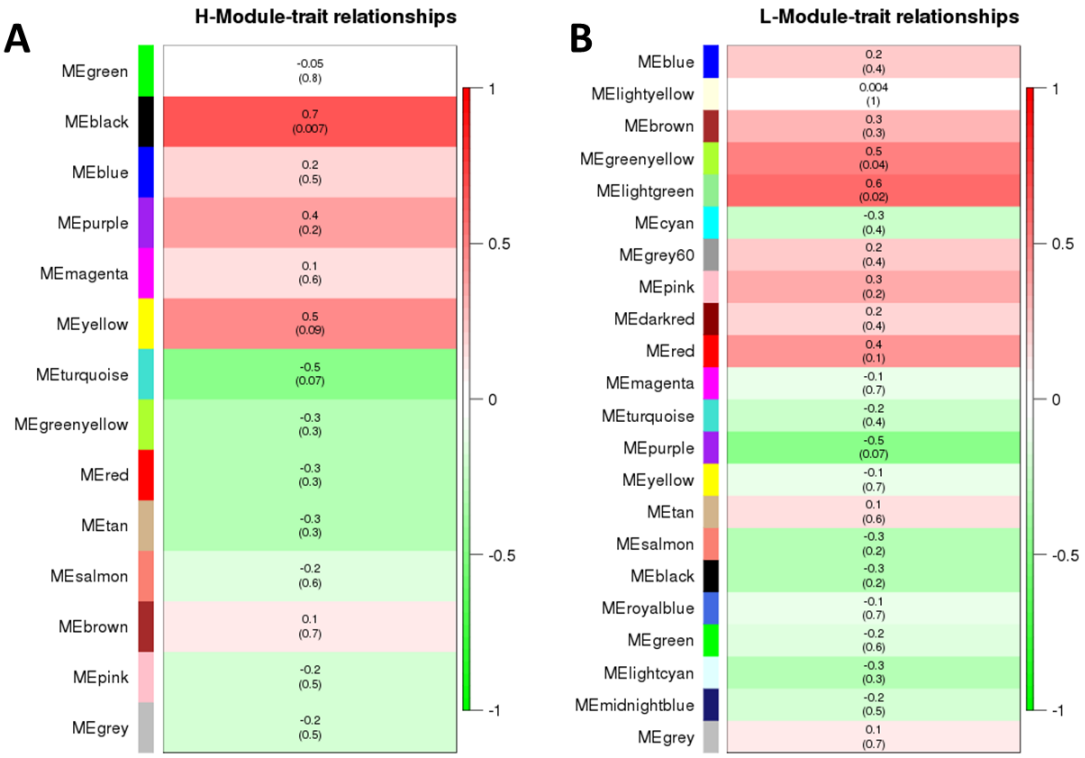

Figure 13. Correlation between miRNA modules and IMF. A: modules identified in High IMF GEBV (H) group. B: modules identified in Low IMF GEBV (L) group. Modules with intense red color have a higher positive correlation (close to +1) and those with an intense green color have a more negative correlation (close to -1).

Table 8. GO terms enrichment of modules significantly correlated with the trait, for mRNA and microRNAs for High (H) and Low (L) groups.

Group	module	corr	pvalue	#molecules	FDR	GO terms
				microRNAs modules		
H	black	0.7	0.007	21	0.045	GO ID 46942:carboxylic acid transport
	yellow	0.5	0.09	25	0.041	GO ID 6417:regulation of translation
	turquoise	-0.5	0.07	105	0.064	GO ID 19377:glycolipid catabolic process
L	greenyellow	0.5	0.04	13	0.007	GO ID 6629:lipid metabolic process
	lightgreen	0.6	0.02	7	0.0001	GO ID 55074:calcium ion homeostasis
	purple	-0.5	0.07	14	0.028	GO ID 19915:lipid storage
				mRNAs modules		
H	darkorange	0.5	0.09	63	0.052	GO ID 42816:vitamin B6 metabolic process
	cyan	-0.5	0.08	187	0.01	GO ID 6954:inflammatory response
	darkgrey	0.5	0.08	83	0.002	GO ID 4117:calmodulin-dependent cyclic-nucleotide phosphodiesterase activity
L	plum1	-0.5	0.06	63	0.053	GO ID 19955:cytokine binding
	magenta	-0.5	0.03	474	0.093	GO ID 2385:mucosal immune response

The darkorange and darkgrey mRNA modules were positively correlated with high IMF content (H group). The genes in these modules were enriched for the GO terms associated with vitamin B6 metabolic process, and calmodulin-dependent cyclic-nucleotide phosphodiesterase activity, respectively. The cyan mRNA module enriched for inflammatory response was negatively correlated with high IMF content. The plum1 and magenta mRNA modules were negatively correlated with low IMF content (L group). The genes in these two modules were enriched for the GO terms cytokine binding and mucosal immune response, respectively.

On the other hand, the black and yellow miRNA modules, that were positively correlated with high IMF content (H group), were enriched for GO terms related to carboxylic acid transport and regulation of translation, respectively. The turquoise miRNA module, which was negatively correlated with IMF content in H group, was enriched for GO terms related to glycolipid catabolic process. The greenyellow and lightgreen miRNAs modules were positively correlated with the low IMF content (L group). These two miRNA modules were enriched for GO terms related to lipid metabolic process and calcium ion homeostasis. The purple miRNA module was negatively correlated with low IMF content and enriched for GO terms related to lipid storage.

2.3 Discussion

The regulation of lipid oxidation and biosynthesis is under strong feedback control in order to maintain homeostasis [27]. Although research studying the influence of miRNAs on metabolism are recent [28], there has been a rapid growth in the number of identified miRNAs that are involved in the regulation of genes and signaling molecules responsible for maintaining lipid homeostasis [20, 29].

2.3.1 Networks enriched for lipid and carbohydrate metabolism

The comparison of miRNA expression between animals with different genetic potential for IMF deposition resulted in the identification of six DE miRNAs involved in fatty acid metabolism and lipid content. The miR-423 and let-7 family, upregulated in the high (H) IMF group, have been previously reported to be associated with obesity related to disorders in glucose metabolism [10, 30] and have been implicated as possible biomarkers for risk of obesity. The miRNAs upregulated in the low (L) IMF group, miR-100, miR-146 and miR-143, have been reported to control aspects of adipogenesis in humans [31-34]. Chen *et al*. [31] suggested that overexpression of mir-143 could promote or inhibit adipogenesis by regulation of MAPK signaling pathway depending on the stage of development. Interestingly, they found that upregulation of miR-143 expression in early stages of adipogenesis blocks adipocyte differentiation, but when it happens later induces clonal expansion of adipose tissue. The upregulation of miR-143 in lower intramuscular fat content animals in this study may be explained by the fact that fat deposition in *Bos indicus* occurs later than that of other species and that these animals were probably slaughtered in early stages of adipogenesis [2, 3, 35, 36].

The enrichment analysis of DE miRNAs' target genes revealed that the PPAR pathway was overrepresented (Table 3 and Fig. 2) in IPA analysis. Furthermore, many important target genes related to lipid metabolism were present in the gene networks identified by IPA (Table 3 and Fig. 1). Peroxisome proliferator-activated receptors (PPARs) are a class of ligand-activated transcription factors that have a well-known influence

on lipid metabolism and glucose homeostasis. PPARs play a role in development of obesity, diabetes and inflammation [37-39]. PPARα regulates transcription of genes involved in lipid β-oxidation, which decreases fat mass and has anti-inflammatory effects, while PPARγ has an opposite effect [40, 41]. The enrichment analysis of DE miRNAs' target genes indicate that lean animals have higher levels of fatty acid oxidation, because genes that lead to lipolysis are targets of downregulated miRNAs in the low (L) IMF group. On the other hand, based on IPA results, miRNAs upregulated in this group would down regulate genes of lipogenesis and adipogenesis.

In order to gain additional insights into the pathways impacted by miRNAs, we performed co-expression analysis by integration of the miRNA and mRNA data. The PCIT analysis revealed that the top negatively DH genes, which had more connections in the low (L) IMF group, were correlated with genes associated mostly with glucose and carbohydrate metabolism (Table 5). The DH genes that may be the most relevant for IMF were SDHAF4 and bta-miR-24. SDHAF4 plays a role in ATP synthesis by the electron transport chain and miR-24 negatively regulates adipocyte differentiation in mice by targeting genes such as FABP4 [42] and hepatic lipid accumulation by downregulating Insig1 [43]. The DE miRNAs bta-miR-143 and bta-miR-146b, upregulated in the low (L) IMF group (Table 2), were both positively correlated with negative DH genes, and were associated with glucose and fatty acids catabolism (Fig. 5). Bta-miR-146b was correlated with almost all negatively DH genes, while bta-miR-143 was only correlated with SDHAF4. This result indicates that DE miRNAs may play a role in co-expression networks that lead to less fat deposition.

The candidate regulatory genes identified by PIF and RIF that negatively regulate IMF deposition were PKM, bta-miR-143 and bta-miR-26b. PKM is associated with glucose metabolism, while bta-miR-26b was related to cholesterol metabolism and lipogenesis [26, 44] (Table 6). The target genes of bta-miR-143 were enriched for glutamate catabolism. Glutamate is a key component in cellular metabolism, and it is related to biosynthesis of lipids, because it is utilized in the citric acid cycle to produce ATP through α-ketoglutarate [45]. MiR-143 downregulates this process by blocking excess ATP production that could induce storage of lipids instead of undergoing lipid degradation. This co-expression analysis reaffirms the importance of the bta-miR-143 in control of fat deposition.

The midnightblue miRNAs module identified by WGCNA in the low (L) IMF group was associated with downregulation of metal ion homeostasis and interleukin-1 beta production and was negatively correlated with four mRNA modules (green, orange, yellowgreen and black) (Fig. 10). These four mRNA modules were enriched for protein kinase B (PKB) signaling cascade, positive regulation of fatty acid oxidation and negative regulation of protein ubiquitination. The PKB signaling pathway promotes protein synthesis and activation of glucose metabolism via insulin regulation. The orangered4 mRNA module was enriched for carbohydrate metabolic process, which is also related to glucose metabolism. It was negatively correlated with the magenta miRNA module, whose target genes are associated with cell differentiation. Thus, our analysis indicates that in animals with lower IMF, glucose signaling and lipolysis are positively correlated with regulation of interleukin production, ion homeostasis and cell differentiation, based on miRNA-mRNA co-expression analysis.

WGCNA revealed that the mRNA module in the high (H) IMF group that was positively correlated with IMF deposition (darkorange) was enriched for vitamin B6 metabolic process (Table 8), which is indirectly related to lipogenesis. Several enzyme reactions involved in fatty acid metabolism require vitamin B-6 as a coenzyme, such as the biosynthesis of sphingolipids [46], which are a class of lipids that are components of cell membranes. Moreover, the black and yellow miRNA modules in the high (H) IMF group were positively

correlated with IMF deposition. The target genes for the miRNA in these modules are associated with carboxylic acid transport and regulation of translation (Table 8). Fatty acids are carboxylic acids and their transport into the mitochondria leads to activation of β-oxidation to produce energy. Thus, it appears that miRNAs in the high (H) IMF group are downregulating genes involved in lipid catabolism. However, the turquoise miRNA module, which was negatively correlated with IMF in the high (H) IMF group, was related to glycolipid catabolic process (Table 8). This indicates that miRNAs associated with high fat deposition are downregulating biological processes such as transport and catabolism of fatty acids, while miRNAs negatively associated with higher fat deposition downregulate glycolipid degradation. Interestingly in the high (H) IMF group, the black miRNA module and cyan mRNA module were negatively correlated with each other (Table 7) and both were differently correlated with IMF, positively and negatively, respectively (Table 8). These miRNAs were associated with downregulation of fatty acid transport, while the mRNAs were associated with inflammation.

In the low (L) IMF group, the greenyellow and lightgreen miRNA modules were positively correlated with IMF. The miRNA in these modules downregulate genes enriched for lipid metabolic process and calcium ion homeostasis, respectively (Table 8). Calcium (Ca) participates in many signaling networks that contribute to modulation of enzyme function, including Ca-sensitive enzymes involved in lipolysis and lipogenesis [47]. The purple miRNA module was negatively correlated with IMF. A majority of miRNAs in this module were expressed at lower levels in lean animals. The target genes of these miRNA were associated with lipid storage. Overall, the miRNAs in co-expression networks associated with low IMF were related to lipid metabolism, lipolysis, lipogenesis and lipid storage.

2.3.2 Networks related to immune system and inflammatory response

It is known that lipid accumulation in obesity activates the immune system which leads to an inflammatory state due to secretion of proinflammatory molecules by adipocytes [48]. Genes associated with inflammatory response were identified as target genes of DE miRNAs that were upregulated in the low (L) IMF group, which was enriched for the PPAR-RXR signaling pathway (Fig. 2). These genes mediate signal transduction from members of the interleukin-1 (IL-1) family. IL-1, which is regulated by PPARα, can induce and regulate a network of proinflammatory cytokines that initiate inflammatory responses [49]. Using the same population of animals as utilized here, Cesar *et al*. [22] previously reported that DE genes were associated with inflammatory response.

The WGCNA results indicated that the cyan and lightgreen mRNA modules in the high (H) IMF group were enriched for inflammatory response and adaptive immune response. They were also negatively correlated with the black and pink miRNA modules (Fig. 11), whose target genes were associated with carboxylic acid transport and positive regulation of leukocyte migration. Moreover, in the low (L) IMF group the target genes of the most connected miRNA module (midnightblue) (Fig. 11) were associated with metal ion homeostasis and regulation of interleukin-1 beta (IL-1B) production. This module was negatively correlated with catabolism of lipids and protein, as described above. This indicates that mRNA and miRNA co-expression networks are involved in pathways that regulate the immune system and inflammation and that they are correlated with lipid and protein metabolism.

The correlation analysis of mRNA and miRNA modules with IMF emphasized that the immune system was related to lipid accumulation (Table 8). The plum1 and magenta mRNA modules in the low (L) IMF group were negatively correlated with IMF and were associated with cytokine binding and mucosal immune response. The cyan mRNA module in the high (H) IMF group was negatively correlated with IMF and was enriched for inflammatory response.

2.3.3 Comparison of Co-expression analysis: PCIT and WGCNA

The hub miRNAs identified in the integrative analysis correlating both the mRNA and miRNA modules were significantly correlated with DH genes in the PCIT analysis. Hub miRNAs (bta-miR-106a, bta-miR-2400, bta-miR-2887, bta-miR-29d-3p and bta-miR-671) in the green module of the high (H) IMF group (Fig. 10) were correlated with positively DH genes, i.e. those with a higher number of connections in the high (H) IMF group. The enrichment analysis of the green miRNA module in WGCNA revealed that the hub miRNAs target genes were associated with ATP synthesis and catabolism of proteins. Green miRNA module was negatively correlated with the pink, blue and brown mRNA modules in high (H) IMF group (Fig. 10), which were enriched for zinc ion binding, glycerophospholipid metabolic processes and angiogenesis. Glycerophospholipids are the main component of biological membranes, derived from esterification of fatty acids [50]. Angiogenesis is influenced by adipogenesis because activated adipocytes produce angiogenic factors for growth [51]. This result indicates that blood vessel growth is positively correlated with energy metabolism and protein catabolism. Both of which are associated with meat tenderness and are positively correlated with marbling [52, 53]. In the DH analysis, the hub miRNAs in green module were correlated with genes involved in regulation of pyruvate metabolism and proteolysis (Fig. 4). This is consistent with previous study where miR-106a was related to promotion of adipogenesis and inhibition of osteogenesis by targeting BMP2 [54].

Hub miRNAs (bta-miR-122, bta-miR-1291, bta-miR-2308, bta-miR-376d, bta-miR-656 and bta-miR-2904) in the midnightblue module from the low (L) IMF group (Fig. 11) were correlated with negatively DH genes, i.e. those with a higher number of connections in the low (L) IMF group. The target genes of the hub miRNAs in midnightblue module were associated with metabolism of metals and interleukin production and were negatively correlated with mRNA modules enriched for regulation of fatty acid oxidation, protein ubiquitination and PKB signaling in low (L) IMF group, as described above (Fig. 11). In the DH analysis, the hub miRNAs in midnightblue module were correlated with genes associated with energy and lipid metabolism and protein ubiquitination (Fig. 3). Of the miRNAs in midnightblue module, two have been previously reported to be important for lipid metabolism, i.e. mir-122 and mir-1291. MiR-122 was first reported as a key regulator of cholesterol and fatty-acid metabolism [55], while miR-1291 regulates glucose transport into the cell [56]. The miR-1291 was a negatively DH gene (Table 4), which demonstrates that WGCNA and PCIT not only identify similar biological processes regulating fat deposition, but they can also identify the same miRNAs in co-expression networks.

2.4 Conclusion

DE and co-expressions analysis indicate that in addition to lipids metabolism, the glucose metabolism and inflammatory response are the main processes involved in IMF deposition. It shows that the miRNAs not only are related to changes in expression between animals with extreme phenotypes for intramuscular fat deposition, but also participate in co-expression networks that affect mRNAs expression and are related to metabolic pathways that can in fact modulate fat deposition. We also identify that both co-expression approached could construct similar miRNAs networks that were correlated with genes and pathways important to phenotype and we have noticed that perhaps lipolysis is prominent in animals with low IMF content rather than lipogenesis in animals with high IMF. This study allowed us to better understand the role of miRNAs regulation and interaction of them to control fat deposition and also revealed new candidate regulatory genes and miRNAs of lipid metabolism.

2.5 Material and methods

2.5.1 Animals and phenotypic data

Genotypic and phenotypic data were collected on 310 Nelore steers sired by 34 unrelated sires that represent the main breeding lineages in Brazilian Nelore from an experimental breeding herd from EMBRAPA between 2009 and 2011 [57]. The animals were raised in feedlots under identical nutrition and handling conditions until slaughter at an average age of 25 months. Samples from *Longissimus dorsi* (LD) muscle located between the 12th and 13th ribs were collected at two time points: at slaughter for RNA sequencing analysis, and 24 hours after slaughter for the intramuscular fat (IMF) content measurement as described below [22].

Approximately 100g of muscle were lyophilized and ground to measure IMF content using an Ankom XT20 extractor and the AOCS procedure (official Procedure Am 5-04) as described Cesar *et al*. [57]. Animals with extreme values for intramuscular fat (IMF) content were selected based on their genomic estimated breeding values (GEBV) [57]. GEBV was predicted by Genomic Best Linear Unbiased Prediction (GBLUP) methodology, which was conducted using ASREML software [58]. A group of 30 animals were selected (fifteen with high IMF GEBV values and fifteen with low IMF GEBV values) for mRNA and miRNA analyses.

2.5.2 RNA extraction and RNA-sequencing

Total RNA was isolated from 100 mg of LD muscle samples from 30 steers with extreme GEBV values. The extraction of total RNA was performed using the Trizol reagent (Invitrogen) according to the protocol described by Chomczynski and Sacchi [59]. After extraction, total RNA was quantified by spectrophotometer (NanoDrop 200 - Thermo Scientific. Wilmington. Delaware, USA). The integrity of the RNA was verified by size separation on a 1% agarose gel and analysis on a Bioanalyzer 2100 (Agilent Technologies - Santa Clara, CA, USA) with the RNA 6000 Nano kit. All samples had an RNA integrity number (RIN) greater than or equal to 8. Then samples were diluted to a final concentration of 200 ng/μL. Sequencing libraries were

generated with the TruSeq® smallRNA Sample Preparation kit (Illumina - San Diego, USA). The concentration of the cDNA libraries was determined with the KAPA Library Quantification Kit (KAPA Biosystems) and then samples were sequenced on a Miseq machine (Illumina), using MiSeq Reagent Kit v3 (150 cycles), generating around 1 million reads/sample.

2.5.3 Reads filtering and miRNAs identification

After sequencing, data quality was evaluated with FastQC [60] and filtered by Phred score quality using FASTX-Toolkit [61] software, where the minimum quality Phred score was 28. Then, the miRDeep2 [62] program was used to identify and quantify miRNAs, using the default parameters. The sequences were mapped against the bovine reference genome *Bos taurus* UMD 3.1 and compared with miRBase database (v. 21) [63].

2.5.4 Differentially expressed miRNAs

In order to identify differentially expressed (DE) miRNAs between the L and H groups, the total count data of each miRNA was analyzed with the DESeq2 package [64], using a statistical model that fitted contemporary group (animal origin and year that the animal enter the experiment) as a categorical fixed effect and age at slaughter of an animal as a covariate. To remove variation due to the preparation of sequencing libraries, the expression data were normalized by library size, as described in the manual of the DESeq2 package [64]. The Benjamini-Hochberg (BH) [65] methodology was used to control the False Discovery Rate (FDR) of DE at 10%.

2.5.5 miRNA target genes and enrichment analysis of DE miRNAs

The miRNA target genes were obtained from the MicroRNA Target Filter tool of QIAGEN's Ingenuity Pathway Analysis (IPA®, Redwood City-CA) that uses TargetScan, miRecords and TarBase as the miRNA target genes databases. After this first approach to obtain the target genes by IPA, the expression of these genes was checked against skeletal muscle RNA-Seq data that had been previously analyzed on the same set of samples [22]. The functional enrichment of target genes was also performed by IPA software to identify enriched metabolic pathways and gene networks associated with lipid metabolism.

2.5.6 PCIT and differential hubbing network analysis

To improve the functional annotation of miRNA and mRNA interactions in a systems biology context, the Partial Correlation with Information Theory (PCIT) analyses [66, 67] were conducted on the combined list of miRNAs (383) and mRNAs (14,650) after normalization of expression level by DESeq2. The miRNAs and mRNAs were filtered to select only those expressed in animals in both H and L IMF groups. The mRNA expression data utilized in this study was previously published by Cesar *et al.* [22].

PCIT was used to evaluate the specific behavior or co-expression between all miRNAs and genes and from this information, differential connectivity or hubbing (DH) [67] was calculated. Differential hubbing is the difference in the number of significant partial correlations (connections) a gene has between two different treatments, in this case compared between H and L groups and filtering those correlations higher than 0.9. BioLayout Express3D [68] software was used to visualize gene networks.

2.5.7 PIF and RIF analysis

To identify putative candidate regulators responsible for the differences observed in phenotypes, the Phenotypic Impact Factor (PIF) and Regulatory Impact Factor (RIF) approaches were performed [17, 66, 69]. PIF gives a 'weight' for the contribution and importance of genes to the differences involved between phenotypes, based exclusively on their numerical properties. RIF is based on differences in the regulator's correlations and it represents the relative importance of genes/miRNAs on the phenotypically relevant part of the network. The RIF1 value is based largely on changes in correlation between two treatments levels (i.e. differential wiring). The RIF2 value allows genes to be ranked as potential regulators based on the expression changes of a regulator and how it can affect the expression of other genes in the network due to treatment differences [17].

2.5.8 WGCNA

The same list of genes and miRNAs used in the PCIT analysis was utilized to run the R package WGCNA [16]. This analysis constructs clusters of highly correlated genes and miRNAs in modules and allows the correlation of them to each other and also to a trait (i.e. IMF content). In contrast to the analysis performed with PCIT, the WGCNA was done separately for genes and then for miRNAs.

Modules of mRNA - In order to construct clusters of genes, pair-wise Pearson correlation coefficients were first calculated between all expressed transcripts to generate a signed similarity. To emphasize (weight) stronger correlations and punish weaker correlations, the signed similarity matrix was then raised to the lowest power β that approximated a scale-free network topology ($R2 > 0.90$) to generate an adjacency matrix [70]. The topological overlap distance calculated from the adjacency matrix is then clustered with the average linkage hierarchical clustering. The default minimum cluster merge height of 0.25 was retained. The clusters created by WGCNA are called modules, and the minimum number of genes in a module was set to 30. Each module represents a group of genes with similar expression profiles across the samples and the expression profile pattern is distinct from those of other modules. Modules were named by a conventional color scheme and genes not classified in a correlated module were grouped in the grey module. After modules were defined, the module Eigengene (MEs) values were calculated. The Eigengene of a module is defined as the eigenvector associated with the first principal component of the expression matrix representing the expression profile of all genes within a given module [16, 71].

Modules of miRNA - The steps for constructing miRNA co-expression modules were as described above. After generating the signed similarity matrix, a power β value was chosen to generate the adjacency

matrix. The topological overlap distance was calculated and a minimum module size of five miRNAs was chosen. Five was chosen as the minimum module size for the miRNAs due to the smaller size of the miRNA transcriptome relative to the mRNA transcriptome [16, 71].

2.5.9 Correlation between mRNA and miRNA modules

An integrative analysis was performed correlating the ME of miRNAs with the ME of mRNAs, for each group. Those modules with a negative correlation higher than -0.4 with a p-value <0.05 were used for enrichment analysis. The co-expression networks among hub miRNAs, representing the whole module, and the GO terms of mRNAs inside the correlated modules were constructed in Cytoscape v.3.3.0 0 [72].

2.5.10 Correlation of modules with trait

Using the ME, the Module-Trait relationships were estimated by calculating the Pearson's correlations between the ME and the animals' phenotypic information (i.e. % IMF) to select potential biologically interesting modules that could explain the phenotypic differences between groups. Modules were selected when they had a p-value of correlation <0.1 with the trait.

2.5.11 MiRNAs target genes and enrichment analysis of co-expression data

The general gene enrichment of GO terms for biological processes was made using BiNGO (Biological Networks Gene Ontology), tool for Cytoscape v.3.3.0 [72] and REVIGO [73] to visualize clusters of GO terms. The Benjamini-Hochberg (BH) [65] methodology was used as a multiple testing correction to control the False Discovery Rate (FDR) at 10%. For miRNAs, the combined results from miRanda and TargetScan approaches were used to identify the target genes and these genes were also filtered by skeletal muscle RNA-seq data of previous study [22] to do the enrichment.

The enrichment of miRNA modules identified by WGCNA was conducted using the target genes information of specific hub miRNAs in each miRNA module. In this case the hub miRNAs were those with the highest Modular Membership (MM) value for the module, which means that these miRNAs have higher connectivity inside the module and are probably more informative [12].

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ANNEX

ANNEX A - Figures 14 and 15 of gene networks enriched in IPA analysis

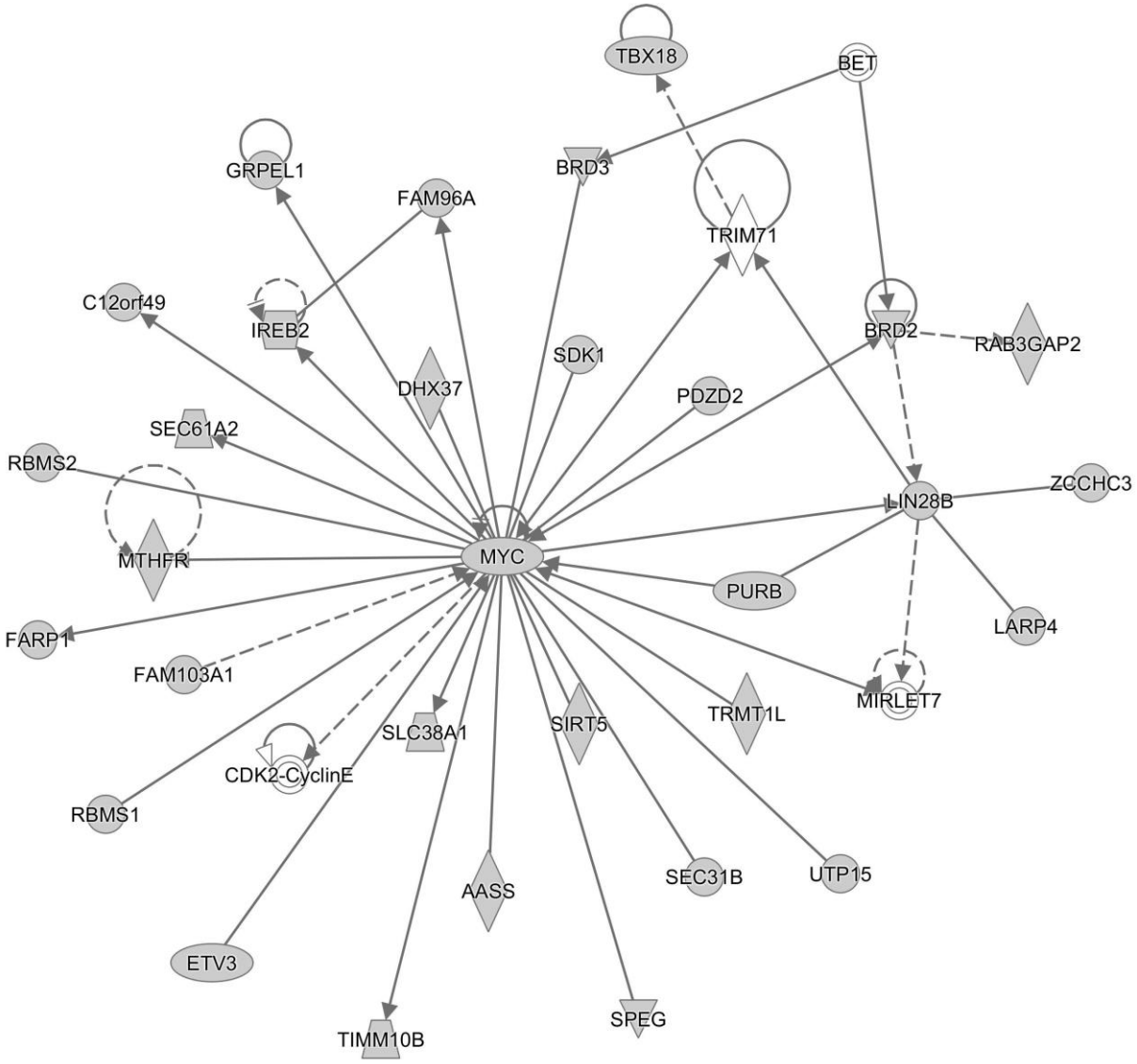

Figure 14. Gene network "Drug Metabolism, Lipid Metabolism, Molecular Transport" identified from the DE miRNA's target genes list generated by IPA. Grey shapes represent target genes and the white shapes are other genes of the network that are not target genes. Solid lines mean direct interaction and dashed lines an indirect interaction between genes.

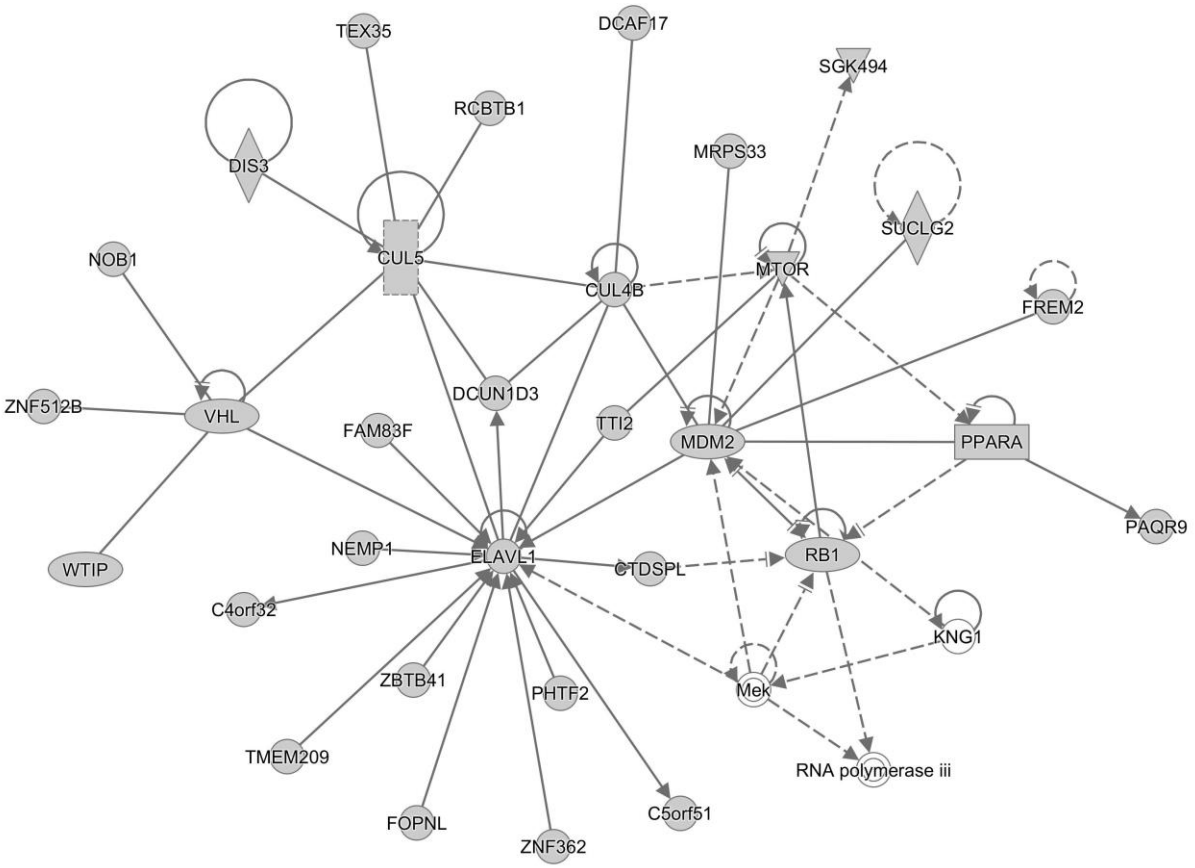

Figure 15. Gene network "Gene Expression, Cell Cycle, Cancer" identified from the DE miRNA's target genes list generated by IPA. Grey shapes represent target genes and the white shapes are other genes of the network that are not target genes. Solid lines mean direct interaction and dashed lines an indirect interaction between genes.

ANNEX B - Figures 16 and 17 of canonical pathways enriched in IPA analysis

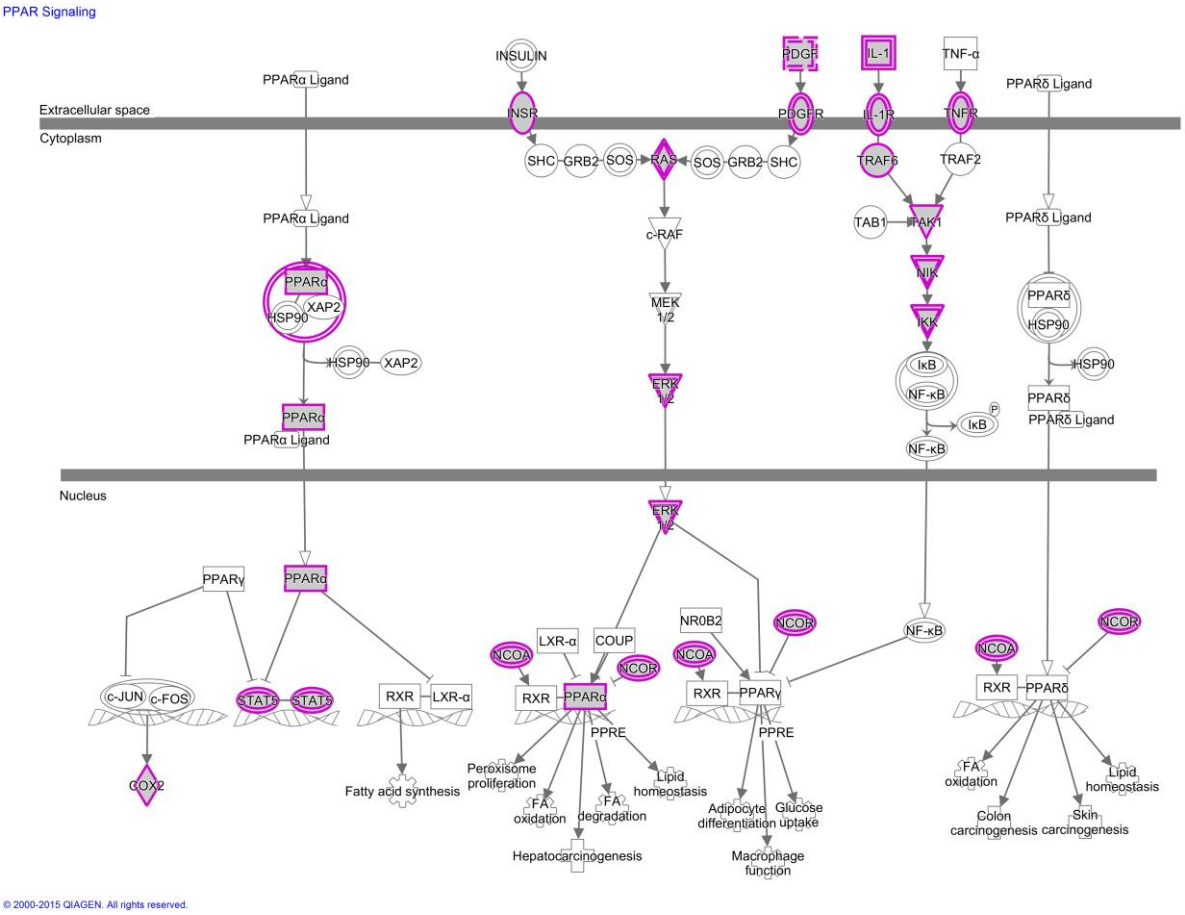

Figure 16. The PPAR Signaling pathway is over-represented in miRNA target genes identified by IPA. The shapes highlighted in purple represent the miRNA target genes and the white shapes represent the other genes of the pathway that are not target genes.

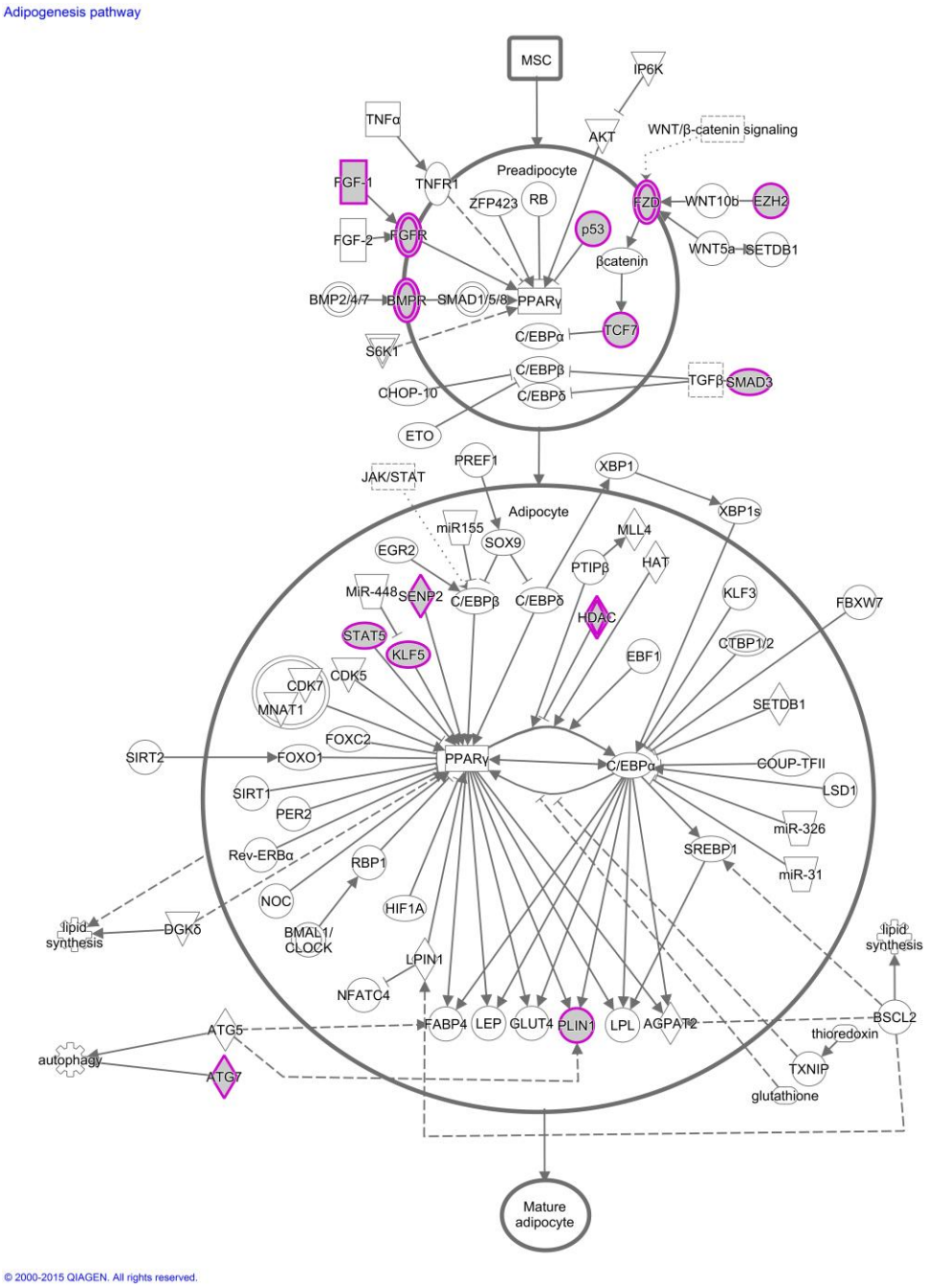

Figure 17. The Adipogenesis pathway is over-represented in miRNA target genes identified by IPA. The shapes highlighted in purple represent the miRNA target genes and the white shapes represent the other genes of the pathway that are not target genes.