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Differentially transcribed genes in skeletal muscle of lambs

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ABSTRACT

The objective of this study was to compare gene transcription profiles in *Longissimus dorsi* muscle of the following four hair sheep genetic groups, Morada Nova (MO), Brazilian Somali (SO), Santa Inês (SI) and ½Dorper × ½Morada Nova (F1). These groups all display different postnatal muscle growth. The transcriptomes of the skeletal muscle of the lambs (at 200 days of age) were profiled by using oligonucleotide microarrays and reverse transcription-quantitative real-time PCR (RT-qPCR). The microarray experiment identified 262 transcripts that were differentially expressed when transcription levels were compared between the different breeds. A total of 23 transcripts among which those involved in skeletal muscle development (MyoD1 and IGFBP4), lipogenesis and adipogenesis (C/EBPδ, PPARγ and PGDS) were differentially expressed in at least in one comparison. Clustering analysis showed that there is greater similarity in gene expression between the MO and SI breeds and between F1 and SO genetic groups. The SO breed has the most distinct expression pattern. The RT-qPCR results confirmed the findings from the microarray study. A positive correlation was observed between the expression of MyoD1 and the cold carcass yield. The negative correlations between the weight and yield of cold carcass with the expression of C/EBPδ mean that the selection for adipogenesis could lead to a lower carcass weight. The GLUT3 and PYGL gene transcripts were negatively correlated with fat thickness, but ATP5G1 was positively correlated with this trait. Interestingly, many genes negatively correlated with PUFA were positively correlated with cold carcass yield. In conclusion, the present work demonstrated that there are breed-specific expression patterns in Brazilian hair sheep genetic groups. The differences in gene expression among genetic groups were consistent with their phenotypic differences. The positive correlation of the MyoD1 expression with the cold carcass yield suggests that this gene is important for tissue growth in sheep. The positive correlation of the C/EBPδ expression with PUFA provides an opportunity to select for lipid deposition in meat animals.

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1. Introduction

The traits that determine productivity in meat animals have their basis in prenatal development and the post-natal growth of skeletal muscle. In particular, the yield of saleable meat and its quality are influenced by growth during the postnatal period (Harper, 1999). Therefore,

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skeletal muscle is a target tissue for identifying candidate genes responsible for these desirable traits (Lin and Hsu, 2005; de Vries et al., 2000). Skeletal muscle growth during the postnatal period occurs through proliferation of satellite cells followed by differentiation and fusion with existing muscle fibres (Moss and LeBlond et al., 1971). Satellite cells are another population of myogenic cells, and these cells also play a role in postnatal skeletal muscle growth and the maintenance of adult muscle fibres.

According to Koohmaraie et al. (2002), animals born with a higher number of muscle cells have greater muscling potential. However, animals in which satellite cells are more active could potentially have the greatest muscle mass. The majority of the DNA content of the muscle is accumulated during postnatal muscle growth and development, which is the direct result of satellite cell activity.

Relatively little is known about postnatal skeletal muscle growth in sheep, and the identification of the genes that play critical roles during this phase is important for improving meat production by selection. In Brazil, the sheep-breeding programs, mainly the one conducted by our group of researchers (Genecoc), aim to meet both the requirements of industry and of consumers. Thus, the selection objectives include maximising the meat quantity and improving the carcass traits (in terms of fat and fatty acids) related to the final product. However, the efficiency of the breeding programs depends on the accuracy with which the individuals undergoing selection are evaluated. Currently, markers or genes have been used to increase the accuracy and response to selection. The identification of key genes involved in specific developmental processes requires an understanding of the global patterns of gene expression in the specific tissue at the specific time of interest. According to Wang et al. (2009), gene profiling provides an effective tool to discover gene expression changes associated with production traits and to discover genes contributing to quantitative variation between breeds of farm animals.

Gene transcript profiling technologies, such as microarrays, allow for genome-wide or tissue-specific examination of global changes in gene transcription. In livestock, microarrays have been used to evaluate the skeletal muscle of pigs (Jiang et al., 2010 and Lin and Hsu, 2005) and cattle (Lehnert et al., 2005; Wang et al., 2009). In sheep, the main studies were designed to evaluate the resistance to gastrointestinal parasites using microarrays with bovine cDNA (Mackinnon et al., 2009; Diez-Tascon, 2005) or ovine cDNA (Keane et al., 2006). Following analysis of gene expression by microarray, Vuocolo et al. (2007) proposed a model depicting the regulatory network and the main epigenetic modifications likely to explain the changes in fibre types and the hypertrophy that characterises the Callipyge phenotype. Bongiorni et al. (2009) analysed the KEGG (www.genome.jp/kegg/pathway.html) pathways among the differentially expressed genes in two milk sheep breeds and identified the molecular differences in milk synthesis during two lactation stages. However, the global transcription profile of skeletal muscle in sheep during growth has not been previously studied.

In this study, we used Sheep Oligo Microarray and reverse transcription-quantitative real-time PCR (RT-qPCR) to compare gene transcription profiles in the Longissimus dorsi of lambs from four different genetic groups (effect of breed-type). These genetic groups display differences in their growth and this study aims to identify genes that may play important roles in the postnatal muscle growth of sheep.

2. Material and methods

2.1. Animals and sample collection

The experiment was conducted using the experimental flock of Embrapa Caprinos e Ovinos (Embrapa Goats and Sheep), Sobral, CE, Brazil. Twenty-four unrelated males lambs of the selected breeds, Morada Nova (MO), Brazilian Somali (SO) and Santa Inês (SI), and of the crossbred ½Dorper × ½Morada Nova (F1), were used in this study. The lambs were born in the same season, were from a single birth and were weaned at an average of 84 days of age. After the weaning, the lambs were raised on an irrigated pasture of Tanzania grass (Panicum maximun Jacq cv. Tanzania) with free access to water and mineral salt. The lambs were free to graze and were supplemented once a day with concentrate (corn and soybean meal) at a rate of 1.5% of body weight. The experiment used a completely randomised design with four treatments representing each genetic group that respected the principles of randomisation, repetition and uniformity of animals and management. The lambs were slaughtered at Embrapa Caprinos e Ovinos facilities at an average of 200.18 ± 7.54 days of age and 20.62 ± 3.46 kg of body weight. The conditions analysed here seek to simulate the present conditions of the production system for lambs in Brazil, where animals are exposed to various challenges such as rain, heat and parasites.

2.2. Carcass evaluation

All animals were weighed immediately before slaughter (the slaughter weight, (SW)). After skinning and evisceration, the carcasses were separated at the tarsometatarsus and carpometacarpal joints and weighed, obtaining the hot carcass weight (HCW) and its yield (HCY=HCW/SW × 100). After 24 hours in cold chamber at 4 °C, the carcasses were weighed again, obtaining the cold carcass weight (CCW) and its yield (CCY=CCW/SW × 100). The Longisimus dorsi (LD) was separated from carcass to obtain its weight. The subcutaneous fat thickness was measured with a calliper rule. Immediately after slaughter (pH 0) and 24 hours post-mortem (pH 24), pH values were taken by a portable pot with electrode penetration (DM 20 - Digmed®) and a resolution of 0.001 pH units. The colorimeter Minolta Chroma Meter CR 300, which was calibrated to the standard, was used to evaluate the colour of the samples. Duplicate samples, with dimensions of 2.5 cm × 1.3 cm × 1.3 cm, were used for the analysis of cooking losses. The lipids from samples were extracted using the methodology presented by Bligh and Dyer (1959). The fatty acids were transmethylated according to method described by Precht and Molketin (2000). The fatty acid profile was determined by gas chromatography according to a method modified from Chilliard et al. (2006). The atherogenic index was calculated as (C12:0+(4*C14:0)+C16:0)/total unsaturated

fatty acids (Chilliard et al., 2003). The indices used to predict the activity of desaturase enzyme (C18:1n9c/(C18:0+C18:1n9c)), (C16:1/(C16:0+C16:1)) and (C18_1n9c+C16_1)/(C18_0+C18_1n9c+C16_0+C16_1)) were also evaluated. The desaturase index is based on the relationship between the substrate and the product for Δ9 desaturase.

2.3. RNA isolation and labelling

Tissue samples from the LD muscle (approximately 3 g) were collected immediately post-slaughter and placed in RNA stabilisation solution (RNA Holder®, BioAgency, São Paulo, Brazil). The tissue samples were kept in the RNA stabilisation solution at 4 °C overnight. The next day, the excess solution was removed, and the samples were stored at −20 °C. The samples were then sent on dry ice to Laboratório de Biotecnologia Animal, at the Universidade Federal de Viçosa for processing. Total RNA was obtained using the RNeasy Miniprep kit (Qiagen Inc., Valencia, CA, USA), according to the instructions of the manufacturer. Purified total RNA samples were stored at −80 °C for microarray experiments. RNA quality and integrity were determined using the Eukaryote Total RNA Nano 6000 assay (Agilent RNA 6000 Nano LabChip® Kit; Agilent Technologies Inc., Santa Clara, CA, USA) on the Agilent Technologies 2100 Bioanalyzer and quantified by measuring A260 nm on a UV/Vis spectrophotometer. Only samples with an RNA Integrity Number (RIN) above 7.0 were used. Fifty nanograms (ng) of total RNA were reverse-transcribed to cDNA with a T7 sequence primer. T7 RNA polymerase-driven RNA synthesis was used for the preparation and labelling of RNA with Cy3 dye (Agilent Technologies Inc., Santa Clara, CA, USA).

RNA spike-in (Agilent RNA Spike-In kit; Agilent Technologies Inc., Santa Clara, CA, USA) controls were used in the reverse transcription reaction to adjust for possible dye effects following the manufacturer's instructions. The spike-in controls represent two sets of ten synthesised RNA mixtures derived from the Adenovirus E1A transcriptome with different concentrations in each set. These spike-in sets were mixed with samples and co-hybridised to arrays. The fluorescent cRNA probes were purified using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) and the mass yields and specific activities of the labelled cRNA targets were determined by measuring the absorbance spectra on a UV/Vis spectrophotometer. Labelled cRNA samples were sent on dry ice to Laboratório de Genômica Pediátrica from the Faculdade de Medicina—Universidade de São Paulo for hybridisation, washing and scanning (described below).

2.4. Microarray experiment design

The main objective of this experiment was to identify which transcripts were differentially expressed among the genetic groups (MO, SO, SI and F1), and an experimental design was used to provide six different comparisons: F1–MO, F1–SI, F1–SO, MO–SI, MO–SO and SI–SO. Six 8 × 15 K slides were used, and each slide (eight arrays per slide for a total of 48 arrays) was considered to be one

block. Therefore, six biological replicates and two technical replicates were provided in each comparison. The total RNA collected from each animal (n=6) from each genetic group (n=4) were hybridised in two slides (technical replicates). Each slide contained all four genetic groups, so the microarray design was completely randomised by block (Supplementary material, Table S-1).

2.5. Hybridisation and scanner

Equal amounts (600 ng) of Cy3 labelled cRNA probes were hybridised onto an 8 × 15 K Sheep Agilent array (GEO accession: G4813A). The Agilent Gene Expression Hybridization Kit (Agilent Technologies Inc., Santa Clara, CA, USA) was used to hybridise fluorescently labelled cRNA to microarrays. The hybridised slides were washed using a commercial kit package (Agilent Technologies Inc., Santa Clara, CA, USA) and scanned using Agilent's DNA microarray scanner. To extract data from the probe, Agilent Feature Extraction Software was used (www.agilent.com/chem/fe).

2.6. Microarray data collection and analysis

Raw data sets were normalised for total fluorescence, which represents the total amount of cRNA hybridised to a microarray. A background correction was applied to the raw data (using the LIMMA normexp+offset 50 method), and the corrected values were normalised (within arrays) using the "quantile" method. After normalisation, the data were log2-transformed. Quality control was performed using a plot density tool. Spots from duplicate probes were averaged, and the normalised data were analysed using R version 2.11.1 (R Development Core Team, 2004) with the MAANOVA package (Bioconduction, R/ MAANOVA). The microarray analysis of variance (MAANOVA; Kerr et al., 2000) for detecting genes with differential expression was implemented. The model used was as follows:

$$Y_{ijk} = \mu + gg_i + s_j + e_{ijk}$$

In this equation, μ is the overall mean, gg_i is the fixed effect of the genetic group ($i=1$–4), s_j is the random effect of the sample ($j=1$–24) and e_{ijk} is the random residual effect.

The t test was used to estimate the significance of the differences for each transcript in each comparison, where $P < 0.05$ was considered significantly different. The Fs statistic, based on the James–Stein shrinkage estimates of the error variance, was used. The false discovery rate was examined using the multiple test adjustment approach (FDR, Benjamini and Hochberg, 1995). The null versions of the test statistics were simulated by 1000 permutations with sample shuffling.

Heat maps and cluster images were developed using the specific functions of R and MAANOVA.

2.7. Reverse transcription-quantitative real-time PCR (RT-qPCR)

Microarray results for the transcript profiling experiments were validated by RT-qPCR on the same cDNA samples used for the microarray analyses. The reactions were performed

using GoTaq® qPCR Master Mix (Promega, Madison, USA), according to the manufacturer's instructions. Each cDNA template for qPCR was reverse-transcribed from 1 μg of skeletal muscle. Complementary DNA was prepared using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA, USA). Twenty sets of PCR primers were designed for those genes (10 genes and two set of primers per gene) with significantly different transcription levels and with functions involving muscle growth or meat quality (Supplementary material, Table S-2). The glucose-6-phosphate dehydrogenase (G6PDH) and succinate dehydrogenase (SDHA) genes were tested (Dervishi et al., 2010) as reference genes. The efficiency of the PCR amplification for each gene was calculated using the standard curve method ($E = 10^{-1/slope} - 1$). The standard curves for each gene were generated by a three-fold serial dilution of pooled cDNA. For this purpose, three cDNA concentrations (50, 75 and 225 ng/μL) and three primers dilutions (100, 200 and 400 nM) were tested. After the analysis of efficiency, we chose sets of primers with the better PCR conditions. The efficiencies of amplification of the target genes and the references genes were similar, ranging from 0.71 to 1.0. The dissociation curves showed no peaks corresponding to primer dimers or nonspecific products for any of the target genes or reference genes. Real-time quantitative PCR measurement for each sample was performed in triplicate in a total volume of 20 μL. The gene expression intensity (measured in cycle thresholds (Ct)) of each gene was normalised according to the transcription level of the reference gene (Ct), and the relative fold-change (ΔCt) was obtained.

2.8. ANOVA and correlation analysis

The values of the gene expression of genes evaluated by RT-qPCR were correlated to those values of expression for the same genes obtained by microarray analysis to validate the differential observed in the microarray experiment.

An ANOVA was performed to verify the differences between the genetic groups for the expression levels obtained by qPCR.

The RT-qPCR values for the expression of these genes in each animal and those predicted by the model cited above in the microarray analysis were correlated with the phenotypic traits of the same animals. These traits were related to growth and carcass (the daily weight gain, slaughter weight, weight of Longissimus dorsi, cold and hot carcass weight and yield, and loin eye area), fat (the amount and thickness of intramuscular fat) and the meat quality (initial and final pH, L*, a* and b* colour, cooking loss, and fatty acids content). Analyses were conducted using the CORR procedure from the SAS Institute Inc. (1996) with Pearson's coefficient ($P < 0.05$).

3. Results

The slaughter weight of SI lambs was greater than the slaughter weight of F1 and MO lambs ($P < 0.05$). MO lambs weighed significantly less at slaughter than lambs of other breeds (SI ≥ F1 ≥ SO > MO; Table 1). The hot and cold carcass weights of SI, SO and F1 lambs were also greater than the hot and cold carcass weights of MO lambs. There is a significant difference in the hot and cold carcass yield between the Somalis lambs and those of other breeds. The intramuscular fat content was significantly different between the genetic groups SI and SO ($P < 0.05$; Table 1). This genetic variability among breeds is important for improvement of lamb growth and meat quality in sheep production.

3.1. Differentially expressed muscle transcripts

To identify which genes were differentially expressed in the LD muscle of lambs, we used the Sheep Oligo Microarray. The genome-wide expression profiling of each element (probe) was assigned to six different comparisons MO–SO (C1), F1–MO (C2), F1–SO (C3), MO–SI (C4), SI-SO (C5) and F1-SI (C6). After the correction for background and the normalisation of the raw data set (48 arrays), the gene expression analyses were performed using 39 arrays. Nine arrays were discarded because of

Table 1

The least-square means values for the slaughter weight (SW), the hot carcass weight (HCW), the cold carcass weight (CCW), the weight of Longissimus dorsi (LD), the hot carcass yield (HCY) and the cold carcass yield (CCY) and intramuscular fat content (IMF) of lambs.

	Genetic group			
	Morada Nova	F1	Santa Inês	Somalis
SW (kg)	14.38 + 1.41[c]	20.82 + 1.31[ab]	23.98 + 0.96[a]	19.54 + 1.31[b]
HCW (kg)	6.20 + 0.78[b]	9.35 + 0.73[a]	10.76 + 0.53[a]	9.20 + 0.73[a]
CCW (kg)	6.04 + 0.77[b]	9.13 + 0.71[a]	10.46 + 0.52[a]	8.99 + 0.71[a]
LD (kg)	0.26 + 0.04[b]	0.35 + 0.04[ab]	0.39 + 0.03[a]	0.39 + 0.04[a]
HCY (%)	42.69 + 0.95[b]	44.55 + 0.88[b]	44.62 + 0.64[b]	47.10 + 0.88[a]
CCY (%)	41.60 + 0.94[b]	43.47 + 0.87[b]	43.38 + 0.64[b]	46.00 + 0.87[a]
IMF (mg/g)	30.20 + 2.65[ab]	45.71 + 3.02[ab]	28.84 + 1.78[b]	58.88 + 3.99[a]
Δ9 desaturase[1]	0.51 + 0.07 b	0.52 + 0.06 b	0.47 + 0.06 b	0.62 + 0.05 a
Δ9 desaturase[2]	0.02 + 0.00b	0.02 + 0.00 b	0.03 + 0.00 b	0.04 + 0.00 a
Δ9 desaturase[3]	0.35 + 0.01 ab	0.37 + 0.01 ab	0.31 + 0.00 b	0.41 + 0.01 a

The means followed by a different letter in the each row are significantly different, as measured by a t test ($P < 0.05$).

[1] Index of the desaturase enzyme = C18_1n9c/(C18_0 + C18_1n9c).

[2] Index of the desaturase enzyme = C16_1/(C16_0 + C16_1).

[3] Index of the desaturase enzyme = (C18_1n9c + C16_1)/(C18_0 + C18_1n9c + C16_0 + C16_1).

inconsistencies in the scanner reading. Discarding these samples did not have a large impact on the analysis because it did not lead to a large unbalance in the experimental design (all six biological replicates were maintained; Table S-1). A total of 262 transcript genes were identified as differentially expressed in the LD muscle between the comparisons. Among these expressed transcripts, 232 were unannotated expression sequence tags (EST), and the BLASTn tool was used to search for annotations of homologous sequences (in *Bos taurus*), which present the same level of nucleotide homology (Supplementary material, Table S-3).

Sufficient information was available for 30 of the transcripts that were identified as differentially expressed among the comparisons. Of these 30 transcripts, seven sequences were redundant; and, we present only 23 transcripts in Table 2. These transcripts were classified

into different categories according to the biological processes in which each is involved. These biological processes include the following: muscle development and growth, adipogenesis and lipogenesis and energy metabolism. Four genes encoding energy metabolic enzymes were differentially transcribed between the comparisons. We also identified one transcript involved in muscle tissue development and one transcript involved in skeletal growth (Table 2). The Somali LD had more transcription of genes encoding muscle satellite cells (*Myogenic differentiation 1*—MyoD1; Table 2) and fatty acid biosynthesis (*Stearoyl-coa desaturase (delta-9-desaturase)*—SCD and *Acetyl-coa acyltransferase 1*—ACAA1; Supplementary material, Table S-3)) compared to MO lambs. Overexpression of the *CCAT/enhancer binding protein delta* (C/EBPδ) transcript was observed in the muscle of MO animals compared to SO. C/EBPδ exhibited a

Table 2
Genes of known function with differential expression in six comparisons (C1–C6).

GenBank ID	Gene symbol	Description	FC-C1[a] MO–SO	FC-C2[2] F1–MO	FC-C3[a] F1–SO	FC-C4[a] MO–SI	FC-C5[a] SI–SO	FC-C6[a] F1–SI
Genes related to skeletal muscle tissue development and growth[b]								
NM001009390	MYOD1	*Myogenic differentiation 1*	**0.45**					
S77394	IGFBP4	*Insulin-like growth factor-binding protein 4*		1.57	1.37	**0.82**		1.29
Genes related to adipogenesis and lipogenesis[b]								
NM001100921	PPARγ	*Peroxisome proliferators activated receptor gamma 1 protein*		1.36	1.33			1.43
DY495847	C/EBPδ	*c/EBP delta gene for CCAT/enhancer binding protein delta*	**2.22**					
NM001009257	PGDS	*Prostaglandin D2 synthase*	0.64					
Genes related to energy metabolism[b]								
NM001009396	ATP5G1LOC443410	*ATP synthase. H+ transporting. mitochondrial F° complex. subunit C1 (subunit 9)*	**0.81**					
NM001024861	PYGL	*Phosphorylase. glycogen*	1.27		1.33	**1.19**		1.25
NM001009770	GLUT3	*Glucose transporter type 3*	**1.17**		1.23	0.82	1.43	**0.86**
NM001009764	GGTA1	*Alpha-1.3-galactosyltransferase*	0.77	1.35			0.84	1.35
Genes related to transcription[b]								
NM001009426	C-MYC	Cellular myelocytomatosis oncogene	**2.81**					
Genes related to chitin catabolic process[b]								
EF581383	LOC100101234	Stabilin-1 interacting chitinase-like protein	**1.36**					
AY392761	LOC443279	Chitinase-3-like protein 1	1.21	0.79			1.16	0.83
Genes related to innate immune response and stress response[b]								
AM231302	TLR6	*Toll-like receptor 6*	**1.08**					
AM167931	CLEC6A	*C-type lectin domain family 6 member A*	**1.06**					
EE783894	VDUP1	*TO-DOWN-G1-4 vitamin D2 up-regulated protein 1*	1.52		1.58		1.40	
Genes related to copper transport[b]								
NM001009429	SAH	*Copper chaperone*	**0.73**					
Genes related to apoptosis[b]								
EE748871	ENDOG	*Endonuclease G*	**0.54**					
Genes related to acute-phase response[b]								
AF180523	FVII	*Factor VIII sequence*	1.06		1.08	1.06		1.08
Genes related to enzyme activator activity[b]								
NM001105261	LOC100125620	*Guanylin precursor*	**1.28**	0.81		**1.11**	**1.11**	**0.90**
Genes related to amino acid metabolism[b]								
AY162433	LAT2	*L-type amino acid transporter subunit LAT2*	1.12	0.89			1.09	0.92
Genes related to signaling[b]								
NM001009752	OTR	Oxytocin receptor	0.92		0.92		0.93	
DQ239650	LOC100037678	TO-UP-C22-8 serine protease 23 precursor	**0.85**		0.74	1.35	0.64	1.17
Genes related to cell adhesion[b]								
U89874	KITLG	*Stem cell factor ou kit ligand*				**1.09**	1.10	**0.94**	1.16

[a] The fold change is defined here as the difference in expression intensity values between the genetic groups – $2^{\binom{\text{difference in Log}}{2}}$ where values > 1 indicate that the first genetic group has a higher expression and values < 1 indicate that the second genetic group has a higher expression. Numeric values in bold correspond to $P < 0.05$, and values not in bold correspond to $P < 0.01$.

[b] GO biological processes according with GeneRIF (Gene Reference Into Function). UniProt (Universal Protein Resource) and DAVID (Database for Annotation. Visualization and Integral Discovery).

2.2-fold higher differential expression in the Morada Nova LD (Table 2). The F1 LD had greater expression of genes *CCAAT/enhancer binding protein (C/EBP) zeta* (C/EBPζ; Table S-3), *peroxisome proliferators activated receptor gamma 1 protein* (PPARγ) and *insulin-like growth factor-binding protein 4* (IGFBP4) than MO, SO and SI lambs (Table 2). Although the muscle of F1 lambs had greater PPARγ and C/EBPζ gene expression than the muscle of SO, SI and MO animals, the intramuscular fat content in these genetic groups did not differ.

The SO animals showed higher expression of genes *ATP synthase. H+ transporting. mitochondrial F° complex. sub-unit C1–subunit 9* (ATP5G1) and *alpha-1.3-galactosyltrans-ferase* (GGTA1) than MO lambs. The ANOVA results were consistent with the microarray analysis for GGTA1, which detected higher gene expression in the genetic groups SO and F1. The *phosphorylase. glycogen* (PYGL) and *glucose transporter type 3* (GLUT-3) transcripts were more highly expressed in the LD of F1 and MO than the SO lambs. The GLUT-3 transcript was more highly expressed in the LD of SI compared with the MO, SO and F1 samples (Table 2).

3.2. Clustering analysis

A clustering analysis and a gene expression heatmap (Fig. 1) was performed for all differentially expressed transcripts and revealed group of genes with similar profiles. The genes were placed into six groups. A set of genes clustered in groups 1 (G1) and 2 (G2) was more highly expressed in MO and SI lambs. Secondly, a set of genes in groups 3 and 4 was more highly expressed in F1

and SO lambs. G1/G2s show higher expression in both MO and SI than F1 and SO, but G3/G4 shows the opposite pattern. The genes clustered in group 5 represent genes of similar expression in all genetic groups. Group G6 contains genes not covered by other groups and displaying similar patterns of expression. The GenBank accession numbers of each of the transcripts grouped into each cluster are shown in Table S-4.

3.3. Validation of the microarray data by RT-qPCR, ANOVA and correlation analysis

RT-qPCR was used to the validate the microarray results. Ten genes were selected because of their functions in muscle growth or energy and lipids metabolism and because transcriptional level differed significantly between genetic groups. Due to low efficiency of amplification by the two pairs of primers for the MyoD1 gene, this gene was excluded from qPCR analysis. The correlation results showed that, overall, there was correspondence between the levels of transcription detected with both techniques (Table 3). The exceptions included four genes (GLUT3, PPARγ, PYGL and C/EBPδ) where the correlation was not significant ($P > 0.05$).

The results of the ANOVA showed that there were significant differences between the transcript expression levels in the genetic groups analysed by qPCR (Table 4). The differences were consistent with those differences observed by the microarray analysis for the genes where the correlation was significant (Table 3).
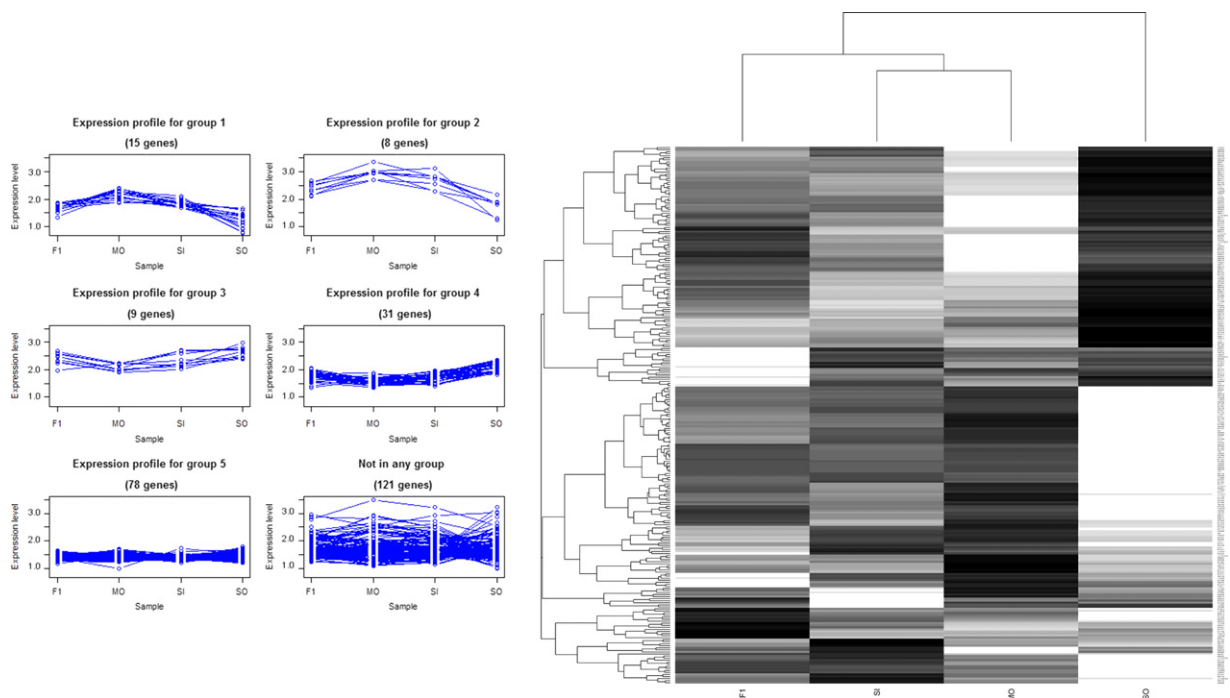

Fig. 1. Patterns of differential expression are depicted. (Right) A hierarchical cluster (heat map) has been constructed with the 262 differentially expressed genes. Genes that are expressed at higher levels are assigned progressively brighter of white in the group, and genes expressed at low levels are assigned shades of black in the group. (Left) The mean value of each genetic group (Morada Nova—MO, Brazilian Somali—SO, Santa Inês—SI and ½ Dorper × ½ Morada Nova—F1) was independently plotted in the graph.

Table 3
Comparison of gene expression measurements by microarray and real-time quantitative PCR (qPCR).

	SCD^M	GLUT3^M	GGTA1^M	PPARγ^M	ATP5G1^M	PYGL^M	IGFBP4^M	PGDS^M	C/EBPδ^M
SCD	0.90 $P < 0.0001$								
GLUT3		0.15 $P = 0.5876$							
GGTA1			0.61 $P = 0.0165$						
PPARγ				0.28 $P = 0.3193$					
ATPG51					0.57 $P = 0.0270$				
PYGL						−0.01 $P = 0.9575$			
IGFBP4							0.75 $P = 0.0014$		
PGDS								0.88 $P < 0.0001$	
C/EBPδ									0.47 $P = 0.0799$

PGDS=prostaglandin D2 synthase; IGFBP4=insulin-like growth factor-binding protein-4; GGTA1=alpha-1.3-galactosyltransferase; PYGL=phosphorylase glycogen; GLUT3=glucose transporter type 3; PPARγ=peroxisome proliferator activated receptor gamma 1 protein; C/EBPδ=c/EBP delta gene for CCAT/enhancer binding protein delta; ATP5G1=ATP synthase. H+ transporting. mitochondrial Fo complex. subunit C1 (subunit 9) and SCD=stearoyl-CoA desaturase.

Table 4
The results of ANOVA tests for gene expression analysis by qPCR.

Gene	Genetic groups			
	F1	MO	SI	SO
SCD	0.232 ab	0.130 b	0.187 b	0.398 a
GLUT3	1.232 a	0.942 ab	0.462b	0.583 a
GGTA1	4.314 ab	3.431 b	3.763 b	6.724 a
PPARγ	0.240 a	0.123 a	0.193 a	0.125 a
ATP5G1	1.638 a	1.260 a	1.752 a	3.137 a
PYGL	1.054 a	1.005 a	0.944 a	1.354 a
IGFBP4	0.352 a	0.156 b	0.232 b	0.245 b
PGDS	1.189 c	1.130 c	2.274 b	3.718 a
C/EBPδ	0.439 a	0.443 a	0.385 a	0.225 a

The means followed by a different letter in each row are significantly different, as measured by a t test ($P < 0.05$); PGDS=prostaglandin D2 synthase; IGFBP4=insulin-like growth factor-binding protein-4; GGTA1=alpha-1.3-galactosyltransferase; PYGL=phosphorylase glycogen; GLUT3=glucose transporter type 3; PPARγ=peroxisome proliferator activated receptor gamma 1 protein; C/EBPδ=c/EBP delta gene for CCAT/enhancer binding protein delta; ATP5G1=ATP synthase. H+ transporting. mitochondrial Fo complex. subunit C1 (subunit 9) and SCD=stearoyl-CoA desaturase.

The correlations between the phenotypic traits and the gene expression from the microarray and qPCR experiments ranged from −0.81 to 0.80 (Table 5). A positive correlation (0.66) was observed between the expression of MyoD1 and the cold carcass yield. The expression of SCD had a negative correlation with the red intensity of the red colour detected in the meat. GLUT3 and PYGL expression were negatively correlated (−0.52 by qPCR and −0.59 by microarray) with the fat thickness, but PYGL was also positively correlated (0.53) with the intramuscular fat content. PYGL, GGTA1 and prostaglandin D2 synthase (PGDS) were all positively correlated (0.57, 0.77 and 0.56) with the cold carcass yield. The expression of C/EBPδ was positively correlated with the total polyunsaturated fatty acid (0.60) but negatively correlated (−0.69 and −0.81) with the cold carcass yield.

4. Discussion

This study represents the first analysis of the global transcriptome by microarray in the skeletal muscle of sheep. The qPCR data analysis confirmed that our microarray data were highly reproducible for the majority of the genes tested.

4.1. The expression of transcripts involved with growth, skeletal muscle tissue, intramuscular fat (IMF) development and energy metabolism

The 2.2-fold higher differential expression of MyoD1 in SO lambs (Table 2) suggests that the proliferative activity of satellite cells, which is the source of new nuclei embedded the muscle fibres, is greater in SO than in MO. Our results suggest that the Somali lambs are more efficient with respect to tissue growth than the Morada Nova lambs during postnatal growth. In fact, it was observed that the SO lambs have a greater LD weight than MO lambs and a better cold and hot carcass yield than all the other genetic groups analysed (Table 1). In pigs, te Pass et al. (2000) suggested that the expression of the MyoD gene family is controlled more by selection for the overall growth rate of the animals than by selection for muscle deposition per se. In Embrapa Caprinos e Ovinos, the process of selection of the genetic groups studied here is focused on the overall growth rate. Rehfeldt et al. (2000, p.183) also suggested that growth selection leads to increases in myoblast and/or satellite cell proliferation rates. Increases in the proliferation rates can be indicated by following factors: higher myonuclear

Table 5

Correlations coefficients for the expression of some genes versus selected phenotype traits related to carcass attributes, fatty acid composition and meat quality of lambs raised in cultivated pasture.

	SCD	GLUT3	GGTA	PPARγ	ATP5G1	PYGL	IGFBP4	PGDS	C/EBPδ	MYOD1
Daily weight gain										(−0.45)
pH0		(**0.80**)	(−0.54)							
L*			−0.56						(**0.67**)	
a*	−**0.67**									
b*			−0.55							
Cooking loss						0.56				
Cold carcass weight									(−0.60)	
Fat thickness		(−0.52)			0.53	−0.59		0.61		
Cold carcass yield			**0.77**		(0.56)	0.57		**0.63**	−**0.69**(−**0.81**)	(0.66)
Intramuscular fat					0.53					
Total MUFA									−0.56	0.40
Total PUFA			−0.55		(−0.6)			−0.55	(0.60)	−0.47
PUFA/SFA			−0.52		(−0.57)			−0.51	(0.56)	−0.44
n6:n3						−0.76			(0.70)	
Essential fatty acids			−0.54		(−0.59)			−0.54	(0.62)	−0.43
n-3					−**0.62**(−0.55)					
n-6			−0.57		(−0.59)			−0.55	(0.60)	−0.47
Atherogenic index	0.61(**0.72**)		0.60		(0.59)					
[1]Δ9 desaturase index		(−0.52)								
[2]Δ9 desaturase index				−0.55				0.54(0.56)		
[3]Δ9 desaturase index		(−0.52)								
C10:0	(0.56)							(0.56)		
C14:0	0.55(**0.65**)		**0.65**		(**0.74**)			**0.67**(**0.73**)	−0.52	
C15:0	0.61(**0.68**)		**0.72**		(0.63)			**0.67**(0.55)	(−0.63)	
C16:0	0.60(**0.71**)		**0.75**		(**0.67**)			0.57(0.63)	−0.51(−**0.67**)	
C16:1				−0.52	(0.59)			**0.63**(0.67)	−0.62(−0.55)	
C17:0					0.56					
C18:0									**0.71**	
C18:1n9c		(−0.52)								
C18:2n6c			−0.54		(−0.57)			−0.52	(0.60)	
C18:2c9T11						−0.51				
C24:0		0.6								
C22:4n6					0.50					

Numeric values in bold correspond to $P < 0.01$, and values not in bold correspond to $P < 0.05$. Values in parentheses are the results of the microarray analysis and values outside the parentheses correspond to the qPCR analysis. [1]C18_1n9c/(C18_0+C18_1n9c); [2]C16_1/(C16_0+C16_1) and [3](C18_1n9c+C16_1)/(C18_0+C18_1n9c+C16_0+C16_1). The following abbreviations are used: SFA=saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA=polyunsaturated fatty acids; UFA=unsaturated fatty acids; n-6=(C18:2n6t+C18:2n6c+C18:3n6+C20:3n6+C20:4n6+C22:4n6); n-3=C18:3n3+C20:3n3+C20:5n3+C22:6n3); atherogenic index=[C12:0+(4*C14:0)+C16:0)/UFA]; $L*$ corresponds to the intensity of lightness, $a*$ corresponds the redness and $b*$ the values for the yellow intensity of meat; PGDS=prostaglandin D2 synthase; IGFBP4=insulin-like growth factor-binding protein-4; GGTA1=alpha-1,3-galactosyltransferase; PYGL=phosphorylase glycogen; GLUT3=glucose transporter type 3; PPARγ=peroxisome proliferator activated receptor gamma 1 protein; C/EBPδ=c/EBP delta gene for CCAT/enhancer binding protein delta; ATP5G1=ATP synthase. H+ transporting. mitochondrial Fo complex. subunit C1 (subunit 9); SCD=stearoyl-CoA desaturase and MYOD1=myogenic differentiation 1.

numbers (e.g., Knizetova et al., 1972; Penney et al., 1983; Brown and Stickland, 1994), higher DNA synthesis rate (Knizetova et al., 1972) and higher total muscle DNA content (e.g., Knizetova et al., 1972; Martin and White, 1979; Fowler et al., 1980; Campion et al., 1982; Jones et al., 1986; Mitchell and Burke, 1995).

The greater expression of SCD gene transcripts in the muscle of SO lambs in relation to the MO lambs suggests a higher activity of this enzyme in SO animals. This hypothesis corresponds with the results of the qPCR analysis (Table 4) and the measurements listed in Table 1 in which Somalis lambs showed the highest index for the desaturase enzyme. This index is used to predict the activity of the stearoyl-CoA desaturase enzyme. However, an assay to measure the SCD activity is needed to confirm this hypothesis. The SCD enzyme catalyses the conversion of the saturated fatty acids into monounsaturated fatty acids (C16:0 to C16:1 and C18:0 to C18:1). Thus, the SCD gene is involved with fatty acid composition. However, no

correlation was found between the mRNA expression levels and levels of monounsaturated fatty acids. Interestingly, genetic groups with higher or lower SCD gene expression have similar contents of IMF (Table 1), which demonstrates that this trait is influenced by other genes and not controlled solely by SCD. Lee et al. (2008) observed high gene expression of SCD in the animals with meat that had a low marbling score. Therefore, the SCD gene may be related to the composition of the fatty acids and not with the total amount of fat.

The MO lambs had higher (compared to the SO lambs) expression of transcripts that are activated at the beginning of preadipocyte differentiation process, such as C/EBPδ. However, they had lower expression of those transcripts expressed at the intermediate period of this process, such as PPARγ. This finding may be an indication that the differentiation of preadipocytes in the MO breed occurs later than in the SO breed. However, an experiment to analyse the various ages at which differentiation occurs

is necessary to confirm this statement. Interestingly, the ANOVA showed no significant difference between the genetics groups with respect to C/EBPδ mRNA expression (Table 4). This result also does not correlate with greater or lesser content of intramuscular fat in the muscle of these animals. The role of the C/EBP family of transcription factors in the preadipocyte differentiation process has been well described in farm animals (Kersten et al., 2000; Wang et al., 2009; Hirwa et al., 2010). Wang et al. (2009) analysed the pattern of expression in the intramuscular fat and observed that the mRNA expression of CCAT/enhancer binding protein beta (C/EBPβ) was relatively greater at early time points. PPARγ displayed a slightly different expression profile, with a peak expression occurring at 25–30 months of age.

The PPARγ gene transcript, which is activated intermittently during adipogenesis by C/EBPδ, was more highly expressed in the muscle of F1 animals. However, the F1 genetic group was similar to other breeds in PPARγ mRNA expression (by qPCR) and IMF, suggesting that this gene was not directly responsible for the amount of fat observed in the animals studied. Although high expression of PPARγ in the LD muscle of crossbred lambs and in the intramuscular fat of cattle has been observed by Byrne et al. (2010) and Wang et al. (2009), respectively, no correlation between IMF and PPARγ was realised or observed by them. PPARγ is mainly associated with adipose tissue and this gene controls adipocyte differentiation and insulin sensitivity (Robinson and Grieve, 2009). Moreover, inducing PPARγ during differentiation is responsible for activating a number of genes involved in the binding, uptake and storage of fatty acids (Wu et al., 1995).

The IGFBP4 gene transcripts were of greater magnitude in the LD of SI lambs compared to the MO lambs. The F1 > SI > MO expression sequence for IGFBP4 is consistent with the observed growth potential of these breeds. This observation corresponds with the function of these proteins because the insulin-like growth factor-binding proteins (IGFBPs) regulate the half-life of circulating IGFs. In addition to their endocrine function, IGFBPs also modulate the IGF availability and the biological activity in local tissues (Duan et al., 2010). IGF is a hormone that regulates growth and cellular metabolism during all of the developmental stages (Davis and Simmen, 2006). In transgenic mice, the over-expression of growth hormone and IGF-I leads to a dramatic enlargement of the skeletal muscle (Frost and Lang, 2003). IGF-I stimulates myoblast and satellite cell proliferation (White and Esser, 1989). IGF-I also stimulates the extension of the hypertrophic and/or proliferative response depending on how the applied selection leads to changes in hormonal system, especially in the growth hormone/insulin-like growth factor-I axis (Rehfeldt et al., 2000). Here, although the genetic groups differ in some traits of weight and carcass yield and expression of IGFBP4, no correlation was found between the expression levels of IGFBP4 and phenotypic traits.

Greater differences in the expression of energy metabolism gene were found between the genetic groups MO and SO. While the muscle of SO lambs had higher expression of ATP5G1 and GGTA1, MO lambs had higher expression of PYGL and GLUT-3. According to Wang et al. (2004), ATP5G1 is a subunit of the ATP synthase family and plays important roles in ATP synthesis, hydrolysis, proton transporting and oxidative phosphorylation (Lu et al., 2002). Of the genes listed above, only PYGL has been previously identified as a candidate gene for the economically important traits we are investigating. The presence of PYGL gene transcripts may indicate higher glycogen content. Glycolytic-type fibres generally contain less intramuscular fat and are implicated in meat aging after slaughter, yielding meat with greater tenderness (Hocquette et al., 1998). Usually, the meat of Brazilian naturalised lambs has less intramuscular fat and a lower rate of muscle mass deposition than the meat of imported breeds. The identification of genes that may increase the selection and genetic gain of these traits is the main focus of the current breeding programs of local breeds. Moreover, the breeds that have undergone genetic progress may be suitable reservoirs of a gene that can promote desirable changes in these traits (Serão et al., 2011).

4.2. Breed-specific expression pattern

The clustering analysis revealed group of genes with similar profiles. The genes IGFBP4, PGDS, PPARγ, GLUT-3, MyoD1, C/EBPδ, GGTA1, PYGL, SCD and ATP5G1 were clustered in the same group (G6), suggesting that they most likely belong within the same metabolic pathway. The interactions between myogenic cells and adipocytes play a significant role in growth and development, including the rate and extent of myogenesis, of muscle growth, of adipogenesis, of lipogenesis/lipolysis, and in the utilisation of energy substrates (Kokta et al., 2004).

The pattern of differential gene expression of the six comparisons provides insights into the biological relevance of the genes and uncovers important trends. The greater similarity of expression between the MO and SI breeds can be explained by origin of the SI breed. This breed was developed from unplanned crosses, which included MO individuals. However, a gene expression heat map (Fig. 1) suggests higher similarities between the MO, SI and F1 genetic groups, with the SO breed presenting a more distinct profile of expression. More differences were observed between the genetic groups MO and SO (Table S-3). The origin of the breeds could also explain this finding. MO and SI are local breeds from Brazil, which originated from Portuguese and Spanish genetic groups and of breeds brought over from Africa with the slave trade. The F1 animals that are ½ Dorper and ½ MO have similarity with the SI, with the MO and also SO breeds. The SO breed is originated from a Black-Head Persian breed, which was brought to Brazil in more recent years (1939). This same breed was the origin of the Dorper breed, which could explain some of the similarities between F1 and SO observed here.

4.3. Correlation between expression levels and phenotypic traits

The correlations between the levels of gene expression and the phenotypic traits were tested because the response

to selection can be predicted from genetic correlation coefficients (Rehfeldt et al., 2000). MyoD1 expression was negatively correlated with the daily weight gain ($r = -0.45$) and positively correlated with the cold carcass yield ($r = 0.66$). These results clearly emphasise the role of MyoD1 in myoblast proliferation and its direct relationship with muscle mass. The cold carcass yield is related to the quantity of meat in the carcass. The negative correlation between MyoD1 and the daily weight gain (GWD) could be justified. The GWD is not only related to muscle mass, but it is also related to fat deposition, growth and the weight of viscera and bones. MyoD is only expressed when satellite cells are activated to proliferate and differentiate into primary myoblasts, which will then differentiate into the cells of the myofibres. The expression of the MyoD1 transcript has a negative relationship with the total proportion of polyunsaturated fatty acids, n6 and essential fatty acids and a positive relationship with monounsaturated fatty acids. This result suggests that the selection for tissue growth may affect the fatty acid profile. Moreover, these results are consistent with the findings of Nsoso et al. (2003) that the selection of the sheep flock for lean tissue growth led to significant increases in lean muscle and decreases in fat. However, the mechanism of how this change occurs needs to be understood, and further studies are necessary.

Due to the negative correlation between the levels of SCD expression and the intensity of red colour in the meat, selection to increase the proportion of monounsaturated fatty acids in meat may lead to a loss in the pigment of the meat and an increase in the saturated fatty acids. The red content reflects the amount of red pigment from myoglobin in the meat, and the appearance is an important parameter of meat quality.

The GLUT3 and PYGL gene transcripts were negatively correlated with fat thickness, but ATP5G1 was positively correlated with this trait.

The correlations observed between C/EBPδ with weight and yield of the cold carcass and PUFA indicate that the selection for a predisposition to develop fat can increase the content of polyunsaturated fatty acids but then decrease the carcass yield. According to Dodson et al. (2010), selective lipid deposition in meat animals is a relatively new strategy for improving the production efficiency while improving the meat quality. Interestingly, many genes correlated positively with PUFA are also negatively correlated with the cold carcass yield.

The levels of expression of PPARγ were not correlated with IMF or fat thickness. As noted by Lee et al. (2008), gene expression of PPARγ might not reflect a positive relationship between the gene expression and the marbling score.

In conclusion, the present study demonstrates that there are breed-specific expression patterns. There were differences in gene expression among the genetic groups upon slaughtering at 200 days of age that were compatible with their phenotypic differences. We identified the following expressed genes in the postnatal muscle of sheep: IGFBP4, PGDS, PPARγ, GLUT-3, MyoD1, C/EBPδ, GGTA1, PYGL, SCD and ATP5G1. The positive correlation of the MyoD1 expression with the cold carcass yield

suggests that this gene is important for tissue growth in sheep. The positive correlation of C/EBPδ expression with PUFA provides an opportunity to select for lipid deposition in meat animals. Using expressed markers or polymorphisms in this gene, which is involved in tissue growth and lipid deposition, may allow for its selection even before the animals begin to express the phenotype.

conflict of interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j. livsci.2012.07.027.

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