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Identification and expression levels of pig miRNAs in skeletal muscle

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ABSTRACT

MicroRNAs are a class of naturally occurring non-coding RNAs. Typically they are ~22 nucleotides long and suppress translation of their targets genes. Several laboratories have attempted to identify miRNAs from pig muscle and the bioinformatics strategies using ESTs have proved to be successful for this aim. In this study we report an *in silico* identification of ncRNA in pig EST libraries focusing on novel pig miRNAs and further investigated the differential expression of pigs miRNAs (known and novel) by quantitative real-time PCR during pre- and postnatal stage from Commercial and local breed Piau pigs skeletal muscle tissue. We identified two miRNAs not yet described in pigs: hsa-miR-1207-5p and hsa-miR-665. Besides, we found 288 target genes for hsa-miR-1207-5p and 214 for hsa-miR-665; from them, four are muscle specific genes. Through expression analyses, differences were found between pre- and postnatal stages and genetics groups. The findings of miRNAs and their muscle-specific targets in pigs will be helpful for understanding the function and processing of this RNA class in the future. Besides, the miRNAs differentially expressed between Commercial and Piau breeds suggest that they can be used to uncover phenotypic differences across different genetic groups.

1. Introduction

MicroRNAs are a class of naturally occurring non-coding RNAs (ncRNA), which are expressed in a wide range of eukaryotic organisms (Bartel, 2004). Typically they are ~22 nucleotides (nt) long and suppress translation by binding to the 3′ untranslated region of their targets genes (Ambros, 2004; Bartel, 2004). Several laboratories have attempted to identify miRNAs from pig muscle (Huang et al., 2008) and the bioinformatics strategies have proved to be successful for this aim. This approach is based on genome sequences or other databases such as expressed sequence tags (ESTs) and genome survey sequences (GSS) which are nucleotide sequences similar to ESTs, with the exception that most of them are genomic in origin, rather than mRNA (Xie et al., 2007; Zhang et al., 2006). Previous research has used the publicly available ESTs to search for new miRNA genes in pigs using the previously known miRNAs from human and mouse and other mammals non-coding sequences (Zhou and Liu, 2010; Seeman et al., 2007).

Recent studies have shown that miRNAs play important gene-regulatory roles in numerous eukaryotic lineages and are often highly conserved across animal

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species. They are involved in many diverse biological processes and may potentially regulate the functions of thousands of genes. The regulation mechanisms can be since repression of translation until cleavage of targeted mRNAs. Also, they may directly target transcription factors which affect animal development and specific genes which control metabolism (Carthew, 2006). Identification of comprehensive sets of miRNAs and other small regulatory RNAs in different organisms is a critical step to facilitate our understanding of genome organization, genome biology and evolution (Carrington and Ambros, 2003), as the different stages of muscle development.

Skeletal muscle development is an important physiological process in animal production, and it directly affects meat production. Muscle mass is mainly determined by muscle fiber number and size in animals. In pigs, muscle fibers are formed in two stages during gestation, including primary and secondary fiber formation, the muscle fiber number being fixed before birth (Wigmore and Stickland, 1983). Investigation of genes expressed during skeletal muscle development is elementary in understanding molecular mechanism of muscle growth for the identification of miRNAs related to muscle specific genes. In this study we report a computational identification of non-coding sequences in owner EST libraries database (Nascimento et al., 2012) focusing on new pig miRNA with their appropriated secondary structure and further investigated differential expression of miRNAs by quantitative real time PCR during pre- and postnatal stage from skeletal muscle tissue in Commercial and local pig breeds.

2. Material and methods

All methods involving animal handling were done in accordance with regulations approved by the institutional animal welfare and ethics/protection commission of the Universidade Federal de Viçosa (UFV; DVT-UFV 02/2008).

2.1. miRNAs and EST dataset

To search potential miRNAs, a total of 2625 previously known miRNAs from *Homo sapiens* (human) and *Mus musculus* (mouse) were obtained from the miRNA Registry Database (Release 16, September 2010; http:/www.mirbase.org; Griffiths-Jones, 2004). The *Sus scrofa* (pig) miRNA dataset was provided by Li et al. (2010) and a total of 777 pig miRNA sequences were obtained. All these sequences were used to construct a mature miRNA reference dataset. In addition, 510,055 ncRNAs sequences were downloaded from fRNAdb, a comprehensive functional RNA database freely available (http://www.ncrna.org/frnadb/), and were used to predict other RNA classes.

All ESTs used in this study were generated from three different pig breeds *semimembranosus* muscle tissue cDNA libraries (Duroc, Large White and a local breed, Piau) (Nascimento et al., 2012). Each EST library was assembled in Contigs and Singlets by CAP3 Sequence Assembly Program (http://deepc2.psi.iastate.edu/aat/cap/cap.html) web server using default parameters (Huang and Madan, 1999), generating unique sequences in each dataset. Besides, we kept short sequences with less than a hundred nucleotides of length for further analysis.

Unique sequences originated by CAP3 program were used for alignment by BlastX program (2.2.18), freely available at (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastNews#1), against UniProtKB/Swiss-Prot protein database (http://www.ebi.ac.uk/swissprot/) using an *E*-value 10 to pick up all no hit sequences. All BlastX non-coding and short sequences, totalizing 2278, were recorded as a distinct dataset and used on BlastN searches to predict known and novel miRNAs.

2.2. Homology searches and secondary structure predictions

The basis for computational identification is the conserved mature miRNA sequence coupled to the predictable secondary structure for its primary miRNA transcript (Ambros et al., 2003; Zhang et al., 2006). We used the mature miRNA reference data set obtained from human, mice and pigs as the subject in Blast searches against pig EST sequences including non-coding sequences and short EST cited before. The BlastN algorithm was used to find homologous miRNA sequences for pig that matched for possible sequence variations by the following parameters: $-W$ 4, $-S$ 1, *F*, *F*, $-q$ -4, $-r$ 5, $-b$ 5 and *E*-value 1.

The sequences which did not have homology with pig miRNA but did with humans and mice were picked up to be mapped into the pig genome. For this, we used the MapMi web server available at http://www.ebi.ac.uk/enright-srv/MapMi/ (Guerra-Assunção and Enright, 2010). Sometimes some could not be mapped, and then we carried out a BlastN of EST sequences against the pig genome database (posted in November, 2010; http://www.ensembl.org/info/data/ftp/index.html) to determinate and confirm their locations in the genome. The BlastN parameters used to map miRNA sequences on pig genome were $-G$ 1, $-E$ 2, $-W$ 15, $-F$, "m D", –*U* and *E*-value 1e−20. EST sequences that matched genomic sequences were selected and the flanking ~60 nucleotides were excised. On this, folding was performed using the program mfold version 3.5 (Markham and Zuker, 2005, 2008) available at http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form web server.

Furthermore, miRNA precursor sequences were considered a potential miRNA when fitting the following criteria: (1) the predicted mature miRNA had no more than four nucleotide substitutions when aligned with known *H. sapiens and M. musculus* mature miRNAs; (2) the RNA sequence folded into an appropriate stem-loop hairpin secondary structure; (3) a mature miRNA sequence site was present in one arm of the hairpin structure and (4) the predicted secondary structure has a negative minimum free folding energy (MFE; ΔG kcal/mol) and 30–70% Adenine+Uracil content (Zhang et al., 2006). These criteria reduced false positives and required that the predicted miRNAs fit the criteria proposed by Ambros et al. (2003). Besides, we submitted the new miRNA precursor sequences to an *ab initio* based program called MiPred (Jiang et al., 2007) which identifies pre-miRNA-like hairpin sequences classifying them as real or pseudo-pre-miRNA. The structures that qualified for the

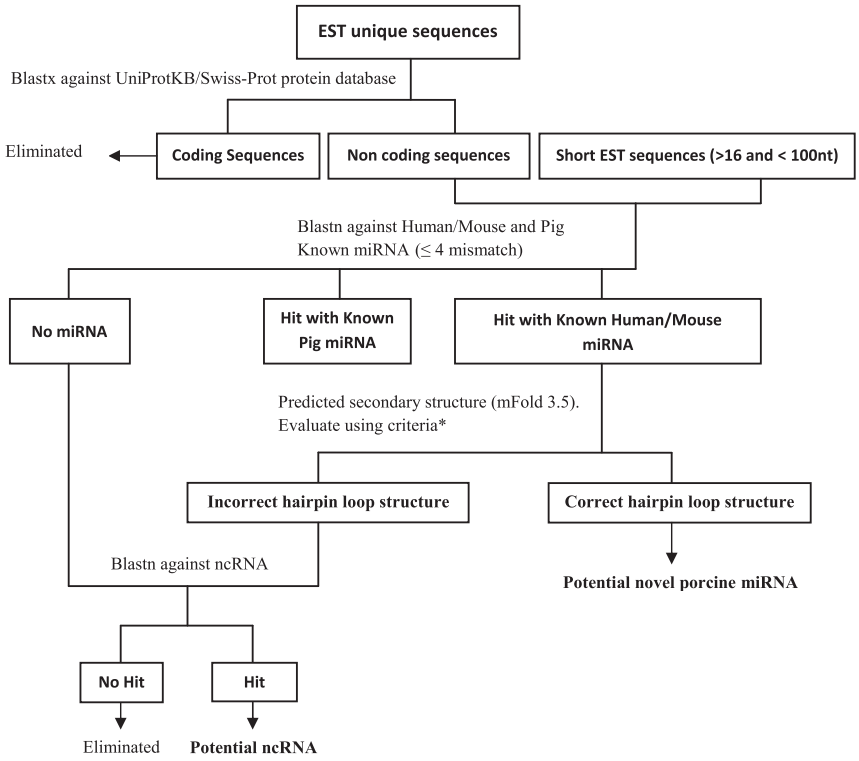

Fig. 1. A schematic diagram of the overall procedure used to identify and analyze miRNA and others ncRNAs. *Modified from Zhang et al. (2006); see text for more details.

succeeding steps were those which were classified as a real pre-miRNA-like hairpin by MiPred program and fully satisfied the criteria proposed by Zhang et al. (2006).

We also employed BlastN searches to obtain pig sequences similar to ncRNAs found in the fRNAdb database using the following parameters: $-r$ 5, $-q$ -4, $-W$ 7, $-G$ 10, $-E$ 6 and *E*-value 1. The steps in the homology search in this study are shown in Fig. 1.

2.3. Phylogenetic analyses

Considering the significance of miRNAs in evolution investigation, the sequences of the predicted pig miRNAs and the known miRNAs in the same family were aligned and phylogenetically analyzed by ClustalW online with default parameters to investigate their evolutionary relationships (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.4. Potential miRNA target prediction

The new miRNAs targets were predicted using the web-based computational software TargetScan, which is freely available at http://www.targetscan.org. *S. scrofa* genes are not included in the current version of TargetScan and the predictions were therefore based on the human mRNA/miRNA interactions. The identified known miRNAs targets were filtered against the TiGER muscle specific genes database available at http://bioinfo.wilmer.jhu.edu/tiger/db_tissue/muscle-index.html.

2.5. Gene network analysis

Aiming to analyze the process of shared pathways, the Ensembl gene identifiers were used. These gene identifiers were obtained from target prediction and other proteins which interact with each other. The programs STRING (Szklarczyk et al., 2011) and TOPPCLUSTER (http://toppcluster.cchmc.org/) were used to obtain the protein–protein interaction and functional Gene Ontology (GO) respectively, identifying the biological mechanisms, pathways and functions involving these genes. The application Cytoscape (www.cytoscape.org/) was used to visualize and edit the identified pathways.

2.6. Quantitative real-time PCR of mature miRNA

Quantitative real-time PCR approach has been massively used on miRNA expression analysis (Chen et al., 2008; Huang et al., 2008; Li et al., 2010). Here, it was used to measure the expression level of miRNA genes during seven different periods of muscle development from two genetic groups as described below.

2.6.1. Tissues samples

A total of 21 *Longissimus dorsi* (LD) muscle samples divided into pre- and postnatal stages were collected for each genetic group (Commercial and local breed Piau). At the UFV Pig Breeding Farm, pregnant Commercial and Piau gilts at 21, 40, 70 and 90 days of gestation were

aborted using the protocol described in Sollero et al. (2011). LD muscle samples were isolated from 12 fetuses for each breed, 21d ($n=3$), 40d ($n=3$), 70d ($n=3$) and 90d ($n=3$), and placed in sterile tubes containing RNA*later*® (Qiagen, Hilden, Germany). Samples were stored at 4 °C overnight and at −70 °C prior to RNA isolation. In the same way, samples of LD from Commercial and Piau castrated males with three different slaughter weights, 30 Kg ($n=3$), 60 Kg ($n=3$) and 90 Kg ($n=3$), were isolated and stored. These time points cover major morphological and physiological changes of pig growth and development throughout pregnancy and days after birth when the pigs reach economical peak weight value.

2.6.2. RNA isolation and reverse transcription

Total RNA from pig LD tissue samples was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentration was determined using the ND-1000 micro-spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA integrity number (RIN) was determined using the RIN algorithm of the Agilent 2100 expert software an RIN ≥ 5 being included in the study. cDNA was synthesized using the miScript Reverse Transcription (RT) Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

2.6.3. Quantitative real-time PCR analysis

The quantitative RT-PCR was performed in a thermal cycler ABIPrism 7300 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA) using miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) and amplification conditions for all systems were 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 15 s, and extension at 60 °C for 60 s. After 40 cycles of amplification, an additional step with a gradual increase in temperature of 60–95 °C was used to obtain the dissociation curve. The best primers and cDNA amplification conditions were used for qRT-PCR analyses. The amplification of target genes was performed at different wells and in duplicates (Pfaffl, 2001; Livak and Schmittgen, 2001). The primers used were published elsewhere (Chen et al., 2008) or obtained from miRNAs identified by Zhou and Liu (2010) or in the present study listed in Table 1.

The experimental design used was completely randomized, with three replicates per period (21, 40, 70 and 90 days of pregnancy; 30, 60 and 90 Kg). Initially, data from qRT-PCR were analyzed using the linear mixed model proposed by Steibel et al. (2009), which is $y_{ijk} = GT_{ik} + D_{ijk} + e_{ijk}$, where y_{ijk} is the measured expression level of gene i on animal j in the treatment k; GT_{ik} is the effect of gene i in the treatment k, each treatment being the combination between the levels of genetic groups (Commercial or Piau) and periods (21, 40, 70 and 90 days of pregnancy; 30, 60 and 90 Kg); D_{ijk} is a random sample-specific effect (common to both genes), $D_{ijk} \sim N(0, \sigma^2_D)$; and e_{ijk} is a residual term, $e_{ijk} \sim N(0, \sigma^2_e)$.

All statistical procedures were performed using SAS® 9.0 for Windows (Statistical Analysis System Institute, Inc., Carry, NC, USA). The macro-QPCR_MIXED (Steibel et al., 2009; https://www.msu.edu/~steibelj/JP_files/QPCR.html) was used to generate commands lines in SAS PROCMIXED in order to analyze qRT-PCR data under a mixed model approach. For each target gene, the comparison of expression values (Ct) of treatment levels (between periods and between genetic groups) was performed using t-student test ($\alpha < 0.05$).

2.7. Clustering relative quantification of gene expression

Hierarchical clustering was performed on PCR data. ΔCt values (target Ct − endogenous Ct) were used in the analysis. Data from four miRNA genes expression values, in both breeds, across seven periods were used for unsupervised hierarchical clustering based on the Complete Linkage method with Pearson Correlation as a distance (Eisen et al., 1998).

3. Results

3.1. Homology searches and secondary structure predictions

For Duroc, LW and Piau datasets we identified respectively 37, 88 and 109 non-coding sequences. Considering also short ESTs, a total of 14 homologous hits to human and/or mouse miRNAs were achieved and mapped to pig genome (Table S1).

From these, two novel potential miRNAs with a real precursor classified by MiPred were confirmed. Their mature sequences were homologous to hsa-miR-1207-5p and hsa-miR-665, hereafter identified as ssc-miR-new1 and ssc-miR-new2. Their mature sequences alignment can be verified in Table 2. The secondary structures of these two novel miRNAs can be viewed in Fig. 2.

Table 1
Primers used for qRT-PCR.

Gene	Mature ID	Primer sequence (5′-3′)	Reference
miR-424	MIMAT0001341	CAGCAGCAATTCATGTTTTGAA	Chen et al. (2008)
miR-133a	MIMAT0000145	TTTGGTCCCCTTCAACCAGCTG	Chen et al. (2008)
hsa-miR-1291[a]	MIMAT0005881	TGGCCCTGACTGAAGACCAGCAG	Zhou and Liu (2010)
hsa-miR-147	MIMAT0000251	GTGTGTGGAAATGCTTCTG	Zhou and Liu (2010)
hsa-miR-1207-5p	MIMAT0005871	TGGCAGGGAGGCTGGGAGGGG	Testing
hsa-miR-665	MIMAT0004952	ACCAGGAGGCTGAGGCCCCT	Testing

[a] Used as an internal control.

Table 2
Alignments of novel putative miRNAs with known miRNAs and their precursor's thermodynamic properties. Regions of mismatch are italicized.

miRNA	EST source[a]	Aligned candidate miRNA sequence (top) against known miRNA (bottom)	MFE (ΔG Kcal/mol)	%A+U content
ssc-miR-new1	Pi_Uf_84A01	UGGCC*A*GGAGG*A*UGGGAGGG*U* (miR-new1) UGGC*A*GGGAGGCUGGGAGGG*G* (hsa-miR-1207-5p)	−40.30	44,66
ssc-miR-new2	Lw_Uf_88G07/Lw_Uf_80F11	ACCAGCAGGCUGAAGCCCC*GG* (miR-new2) ACCAGGAGGCUGAGGCCCC*CU* (hsa-miR-665)	−44.70	34.78

[a] cDNA from sequence in which the miRNA was identified; Pi and Lw mean that these sequences are from local Piau breed and Large White breed libraries respectively.

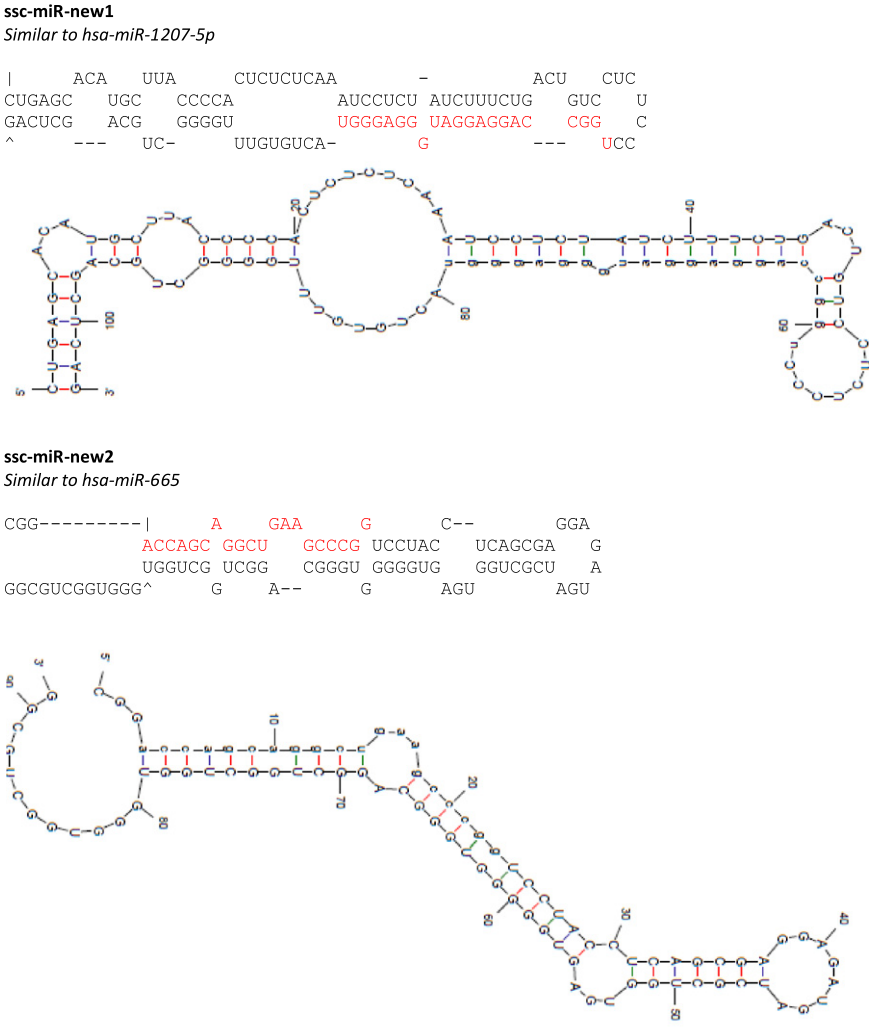

Fig. 2. Hairpin secondary structures predicted by mFold 3.5 for the novel putative miRNAs with the mature miRNA sequences in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A substantial number of remaining no hit sequences showed homology with non-coding sequences. A total of 1231 non-coding sequences were Blasted having 486 (39.5%) similarities with other known ncRNA sequences as we can see in Table S2.

3.2. Phylogenetic analyses

miRNA sequence comparisons across miRNA families showed that the two mature miRNAs of ssc-miR-new1 and ssc-miR-new2 had a high degree of sequence

similarity with the other members (Table 3) performed by ClustalW program.

3.3. Potential miRNA target prediction

Sus scrofa genes are not included in the current version of TargetScan and the predictions were therefore based on the human mRNA/miRNA interactions. A total of 503 potential miRNA targets were found for the two new miRNAs and both revealed target multiplicity. The miRNA which had more number of targets was the hsa-miR-1207-5p with 289, while hsa-miR-665 had 214 targets.

Table 3
Comparisons of sequence similarity between the pig miRNAs and corresponding miRNA family members; the similarity values are given by ClustalW program score.

EST source*	miRNA	Similarity
		Mature sequence
Pi_Uf_84A01	hsa-miR-1207-5p	80.0
Lw_Uf_88G07/Lw_Uf_80F11	hsa-miR-665	80.0

* cDNA sequences in which the miRNA was identified; Pi and Lw mean that these sequences are from local Piau breed and Large White breed libraries respectively.

Four muscle specific targets were identified. For miRNA hsa-miR-1207-5p three genes were identified (CAMK2D, AK1 and SLC25A34) and just one target was identified for the hsa-miR-665 (DIDO1).

3.4. Gene networks analyses

It was possible to identify 13 genes with obvious roles in muscle physiology allowing an insertion of 12 into a relevant functional metabolic network (see Fig. 3); the SLC25A34 target gene was not connected to it. This major network is composed of five secondary connected networks which are kinase activity, programmed cell death, cell projection, synaptic transmission and genes involved in activation of NMDA receptor upon glutamate binding and postsynaptic events pathway. Table S3 describes the function of the 12 genes which are present on the network plus SLC25A34 gene using the assignment of GO terms.

3.5. Quantitative real-time PCR of mature miRNA

The efficiencies of amplification by qRT-PCR represented around 100% in each cycle, and the relative abundance was calculated using an equation to correct differences in efficiency as described by Pfaffl (2001). A housekeeping gene such as the HPRT1, commonly used as

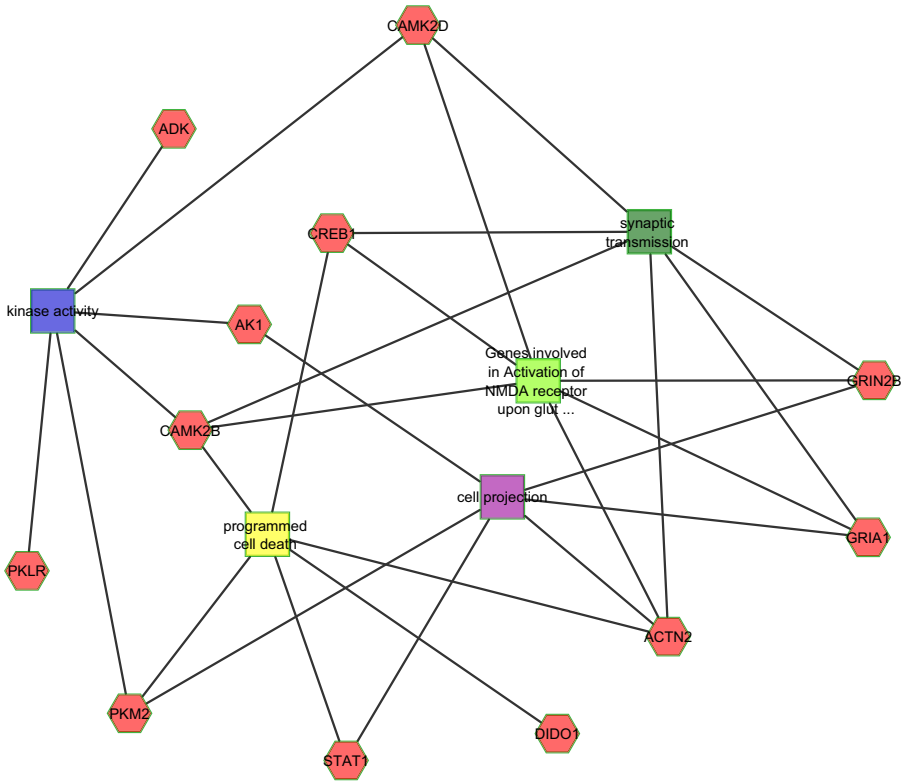

Fig. 3. Functional gene networks and their interactions. It describes the relationships between three miRNAs target genes (AK1 and CAMK2D being hsa-miR-1207-5p target genes and DIDO1 being hsa-miR-665 target gene) with nine linked genes (in red) and five important subnets: kinase activity (in blue), programmed cell death (in yellow), synaptic transmission (in dark green), cell projection (in purple) and genes involved in activation of NMDA receptor upon glutamate binding and postsynaptic events (in light green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

an internal control for such analysis, was not suitable for normalization in these experiments because its transcription was altered across the periods. The hsa-miR-1291, which was consistently expressed in our study according to geNorm program (Vandesompele et al., 2002), was therefore used as an internal control. We analyzed the expression level of three known miRNAs (miR-424, miR-133a and hsa-miR-147) and two novels in pigs (hsa-miR-1207-5p and hsa-miR-665) across seven life time periods for Commercial and Piau samples. From the two novel miRNAs identified in this work, only the hsa-miR-665 amplified efficiently in both breeds; the hsa-miR-1207-5p amplified only in Piau breed during postnatal stage.

Unsupervised hierarchical clustering based on the gene expression values (ΔCt) in both breeds across all seven periods showed the miRNAs expression levels (displayed as a heat map in Fig. 4). The probability values for each contrast (gene/period versus period) demonstrate the significance of each contrast ($p < 0.05$) (Table S4). Besides, the miRNAs relative expression values between Commercial and Piau breeds for all seven periods are shown in Fig. 5.

4. Discussion

We sought to identify novel miRNA genes across the unclassified sequences in our cDNA libraries (Nascimento et al., 2012) which were shown to be a useful source for this aim. The property of miRNAs to be highly conserved across closely related species was used in order to predict novel pig miRNA candidates (Kim et al., 2006). Here we suggest the discovery of two novel miRNAs similar to hsa-miR-1207-5p and hsa-miR-665 by computational approaches following the criteria proposed by Ambros et al. (2003).

From the total of targets identified here for hsa-miR-1207-5p and hsa-miR-665, four were muscle specific: AK1, CAMK2D and SLC25A34 which are regulated by hsa-miR-1207-5p and DIDO1, regulated by hsa-miR-665. The AK1 encodes for adenylate kinase gene, which is an enzyme involved in the adenine nucleotide composition regulating within a cell. Hittel et al. (2005) related a significant increase in the abundance and activity of AK1 in muscle from obese/overweight and morbidly obese women. Here, this gene was identified as a target for the miRNA which had similarity with local breed Piau EST, this breed being known for its ability to produce a large amount of fat.

For the same miRNA we have two other target genes (CAMK2D and SLC25A34). The CAMK2D, which is a calcium/calmodulin-dependent protein kinase type II delta chain, is a multifunctional protein kinase, with complex structural and autoregulatory properties. Evidence suggests a structural diversity in CaMKII isoform variants, as an important determinant of cellular function (Hudmon and Schulman, 2002; Bayer and Schulman, 2001). The SLC25A34 is a member of solute carrier family 25, which consists of proteins with function as transporters of a large variety of molecules (Pebay-Peyroula et al., 2003). All these molecules originate from macromolecules that constitute the energy sources of cells and which are broken down into less complex molecules by cellular enzymes (Palmieri, 2004). The fourth gene, DIDO1, is a target of hsa-miR-665, and encodes for death inducer-obliterator 1 protein, which is identified as a gene up-regulated early in apoptosis by several stimuli. The over-expression of DIDO-1 in cells induces massive apoptosis without any apoptotic stimuli (Garcia-Domingo et al., 1999).

Among these four identified genes, three (CANK2D, AK1 and DIDO1) and nine others (ADK, PKLR, PKM2, STAT1, CAMK2B, CREB1, GRIN2B, GRIA1 and ACTN2) were grouped into a network of functional relevance. All

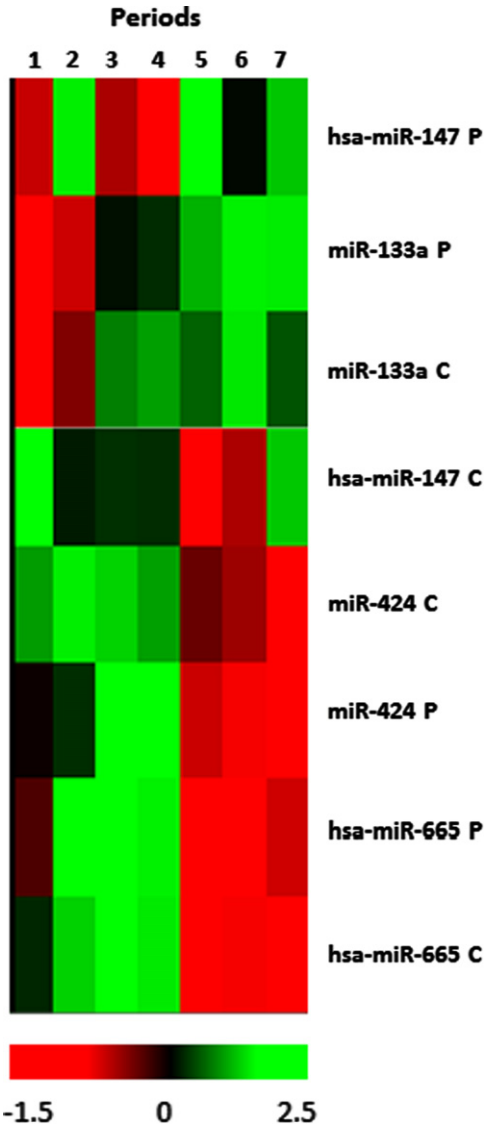

Fig. 4. Unsupervised hierarchical clustering of miRNAs expression levels across all seven periods (1–4 prenatal, 21, 40, 70 and 90 days of pregnancy respectively and 5–7 postnatal, 30 Kg, 60 Kg and 90 Kg slaughter weight) in Commercial (C) and Piau (P). They showed similar expression and a median expression value equal to zero was designated in black; green increased expression and red reduced expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

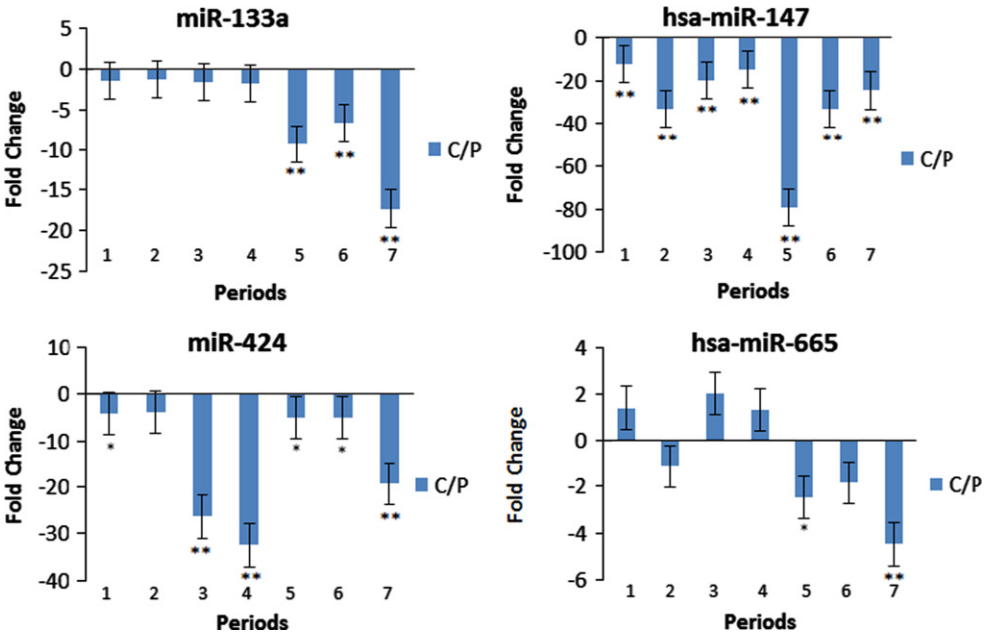

Fig. 5. Relative expression (fold change) value between genetic groups (C/P=Commercial line/Piau) within each period for each miRNA gene. $^{*}p < 0.1$; $^{**}p < 0.001$.

proteins are connected with each other by the subnets kinase activity, synaptic transmission, cell projection, programmed cell death and genes involved in activation of NMDA receptor upon glutamate binding and postsynaptic events. The NMDA or N-methyl-D-aspartate receptors are glutamate-gated ion channels widely expressed in the central nervous system which play key roles in excitatory synaptic transmission (Dingledine et al., 1999).

The sequences which did not have similarity with miRNAs were aligned against other ncRNAs. Most of the known ncRNAs fulfilled relatively generic functions in cells, such as the ribosomal RNA (rRNAs) and transfer RNA (tRNAs) involved in mRNA translation; spliceosomal RNAs or small nuclear RNAs (snRNAs) involved in splicing and small nucleolar RNAs (snoRNAs) involved in the modification of rRNAs (Mattick and Makunin, 2006) were also detected.

For a better understanding of miRNAs biological role, we performed a qRT-PCR across seven periods (four prenatal and three postnatal) in two genetics groups for three known miRNAs and two novels. The miRNAs miR-424 and miR-133a were described by Chen et al. (2008) when they had their expression levels measured during two muscle development periods (65 and 95 days of pregnancy) in Landrace pig breed. Their results showed that miR-424 is down-regulated while miR-133a is up-regulated across these two periods. Our results did not find this difference of expression level during this time. We observed a higher expression during prenatal stage for the gene miR-424 in both breeds (Fig. 4) with it being more expressed in Piau animals (Fig. 5). miR-133a was lower expressed at periods 1 and 2 getting higher from the 70th day of pregnancy until 90 Kg time point again (Fig. 4) being more expressed in Piau animals during postnatal stage (Fig. 5). Other miRNA analyzed here was discovered in pigs by Zhou and Liu (2010) (hsa-miR-147). They collected pig mRNAs from the NCBI GenBank nucleotide database and searched potential miRNAs based on similarity. The expression level in the skeletal muscle of this miRNA showed a differential between pre- and postnatal stages being more expressed during most of the prenatal stage, having high level again at 90 Kg time point in Commercial animals. In Piau animals its expression was shown to be higher at 40 days of pregnancy getting down until the first postnatal stage, showing a high expression during the postnatal stage (Fig. 4). When this gene is compared between breeds it was significantly more expressed in Piau animals in all periods (Fig. 5). This expression in the skeletal muscle has not been reported in the consulted literature. But, our results show that its expression was statistically different between pre- and postnatal stages (Table S4) being more expressed during prenatal stage in Commercial animals. Nevertheless in Piau animals, even having been more expressed than Commercial animals during all periods, its expression did not vary statistically between periods (Table S4). More studies are necessary about this new pig miRNA and its function in myogenesis.

From those new pig miRNAs identified here only the hsa-miR-665 could be amplified in all periods. The hsa-miR-1207-5p was expressed only during postnatal stage in local breed Piau. The hsa-miR-665 expression levels during the mentioned periods showed a high expression during prenatal stage, when a peak during 70 and 90 days takes place. This period is known to be related with the second generation of muscle fiber formation which is between 54 and 90 days of pregnancy (Wigmore and Stickland, 1983). At 30 Kg time point of the postnatal

stage, this expression decreases, as an evidence that this gene is highly expressed after the first generation of muscle formation (35–54 days of pregnancy) until birth, when it starts to be low, in both breeds. By this way, adding the information about their muscle target genes network, the new pig miRNAs identified here as well as their relation with muscle development may be studied more.

5. Conclusion

This work suggests that EST analysis is a feasible strategy for identifying ncRNAs as novel miRNA candidates. In the current study, the identification of two new pig miRNAs and other known non-coding RNAs was proposed through the homology search pipeline. The findings of miRNAs and their muscle-specific targets with their metabolic network are helpful for understanding the function and processing of pigs small RNAs in the future. Furthermore, the expression level of the miRNAs analyzed across pre- and postnatal stages and between two genetic groups (Commercial and local breed Piau) demonstrates a role in biological events during skeletal muscle development and may be worthy of further investigation on the biological role in pigs. Besides, the miRNAs differentially expressed between Commercial and Piau breeds suggest that they can be used for more studies of phenotypic differences between these two breeds.

Conflict of interest statement

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.2013.02.019.

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