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1 *Article*

2 Examining the genetic background of porcine muscle 3 growth and development based on transcriptome and 4 miRNAome data

5 **Katarzyna Ropka-Molik[1,*], Klaudia Pawlina-Tyszko[1], Kacper Żukowski[2], Katarzyna Piórkowska[1],**
6 **Grzegorz Żak[2], Artur Gurgul [1], Natalia Derebecka [3], Joanna Wesoły [3]**

7 [1] Department of Animal Molecular Biology, National Research Institute of Animal Production, Krakowska
8 1, 32-083 Balice; Poland
9 [2] Department of Cattle Breeding, National Research Institute of Animal Production, Krakowska 1, 32-083
10 Balice; Poland
11 [3] Laboratory of High Throughput Technologies, Institute of Molecular Biology and Biotechnology, Faculty
12 of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań; Poland
13 * Corresponding author: Katarzyna Ropka-Molik, email: katarzyna.ropka@izoo.krakow.pl, Laboratory of
14 Genomics, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice, Poland, telephone
15 +48 666 081 208; fax +48 012 285 67 33.

16 **Abstract:** Recently, selection in pigs has been focused on improving the lean meat content in
17 carcasses; this focus has been most evident in breeds constituting a paternal component in breeding.
18 Such sire-breeds are used to improve the meat quantity of cross-breed pig lines. However, even in
19 one breed, a significant variation in the meatiness level can be observed. In the present study, the
20 comprehensive analysis of genes and microRNA expression profiles in porcine muscle tissue was
21 applied to identify the genetic background of meat content. The comparison was performed
22 between whole gene expression and miRNA profiles of muscle tissue collected from two sire-line
23 pig breeds (Piertain, Hampshire). The RNA-seq approach allowed the identification of 627 and 416
24 differentially expressed genes (DEGs) between pig groups differing in terms of loin weight between
25 Pietrain and Hampshire breeds, respectively. The comparison of miRNA profiles showed
26 differential expression of 57 microRNAs for Hampshire and 34 miRNAs for Pietrain pigs. Next, 43
27 genes and 18 miRNAs were selected as differentially expressed in both breeds and potentially
28 related to muscle development. According to Gene Ontology analysis, identified DEGs and
29 microRNAs were involved in the regulation of the cell cycle, fatty acid biosynthesis and regulation
30 of the actin cytoskeleton. The most deregulated pathways dependent on muscle mass were the
31 Hippo signalling pathway connected with the TGF-beta signalling pathway and controlling organ
32 size via the regulation of ubiquitin-mediated proteolysis, cell proliferation and apoptosis. The
33 identified target genes were also involved in pathways such as the FoxO signalling pathway,
34 signalling pathways regulating pluripotency of stem cells and the PI3K-Akt signalling pathway. The
35 obtained results indicate molecular mechanisms controlling porcine muscle growth and
36 development. Identified genes (*SOX2, SIRT1, KLF4, PAX6* and genes belonging to the transforming
37 growth factor beta superfamily) could be considered candidate genes for determining muscle mass
38 in pigs.

39 **Key words:** RNA-seq; miRNA; pig; NGS; transcript analysis; muscle
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41 1. Introduction

42 In pigs, as one of the major domesticated meat animals, processes regulating muscle growth and
43 development have been widely investigated. However, despite numerous studies focused on the
44 regulation of skeletal muscle growth and performed on various breeds, the obtained findings did not
45 provide a clear view of the process. The next-generation sequencing (NGS) technology provides new

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possibilities for gene expression measurements and can be helpful to identify the genetic background of phenotypic traits [1].

In pigs, the analysis of global gene expression profiles using NGS technology in *semimembranosus* and *longissimus dorsi muscles* indicated genes potentially related to muscle growth [2, 3]. Furthermore, the global gene expression profile was also analysed in terms of porcine meat quality [4]. Authors compared muscle transcriptomes of three pig breeds (Pietrain, Polish Landrace, and Pulawska pig) characterized by different production traits and selected genes potentially being under long-term selection focusing on improving the meat content in carcasses. Other studies confirmed the significant role of miRNAs in skeletal muscle growth and development as well as muscle disorders (atrophies and myopathies) [5-7]. The comparison of miRNA profiles between prenatal and postnatal ontogenesis periods allowed us to detect miRNAs and their muscle-specific targets associated with muscle development [8]. Tang et al. [9], performing a comprehensive analysis of miRNA-mRNA profiles in muscle tissue of local pig breeds, indicated the set of genes and miRNAs critical to prenatal skeletal muscle development.

A broad range of miRNA effects on gene expression and the translation process indicate the necessity of a comprehensive analysis of global gene and miRNA expression profiles. Such an approach will allow the detection of mechanisms responsible for gene expression regulation in the processes of muscle growth and development. The aim of the present research was to comprehensively identify genes and miRNAs potentially related to muscle growth in pig species. The RNA-seq method was applied to compare whole gene expression and miRNA profiles of muscle tissue collected from two sire-line pig breeds, which are characterized by high muscularity but differ in terms of this feature within each breed.

2. Materials and methods

2.1. Animals

RNA-seq and miRNAome analyses were performed on *longissimus lumborum* samples collected from 13 pigs representing two sire-line breeds – Pietrain and Hampshire. Animals were maintained at the same housing and feeding conditions according to the procedure previously described [10]. When animals reached a weight of 105 kg (±2.5), they were slaughtered, and after 24 h of chilling, the half carcasses were dissected. Based on dissection data, pigs were selected from a larger population in terms of weight of loin muscle to obtain groups with possibly extreme phenotypes in each breed (Table 1). Pigs included in the present study were unrelated (minimally three crosses back).

Immediately after slaughter, the tissue samples were collected into the RNAlater solution (Ambion, Thermo Fisher,) and stored at -20°C. Total RNA was isolated from muscle tissue using Direct-zol RNA Mini Prep (Zymo Research, TK Biotech, Poland). The quality and quantity of the obtained RNA were checked using a NanoDrop 2000 spectrophotometer (Thermo Scientific; Wilmington, USA) and a TapeStation 2200 system (RNAScreen Tape, Agilent, Perlan Technologies, Poland). Samples with RIN values above 7.5 were used to construct libraries.

Table 1. The differences in muscle weight observed between the analysed groups for both breeds.

	Hampshire						Pietrain					
	Low (n=3)			High (n=3)			Low (n=3)			High (n=3)		
Weight of loin (kg)	54.0	±2.80	**b**	63.4	±2.05	**a**	57.9	±1.27	**B**	64.3	±0.41	**A**
Lean meat percentage	63.3	±1.73		66.1	±0.35		68.1	±2.45		69.8	±2.5	
Weight of ham (kg)	8.69	±0.31	**b**	10.37	±0.25	**a**	9.51	±0.54		9.83	±0.49	

Data are presented as means± standard error; means with different letters differ significantly a,b – pvalue 0.05; A,B – pvalue 0.01).

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87 *2.2. Transcriptome sequencing and data analysis*

88 The cDNA libraries were constructed from 300 ng of total RNA with the TruSeq RNA Sample
89 Prep Kit v2 kit (Illumina) according to the protocol. The quantification of obtained libraries was
90 performed with Qubit 2.0 Fluorometer (Invitrogen, Life Technologies) and TapeStation 2200
91 instrument (D1000 ScreenTape; Agilent). The mRNA sequencing was performed in 101 single-end
92 run on a HiScanSQ platform (Illumina) with the use of TruSeq SR Cluster Kit v3- CBOT-HS and
93 TruSeq SBS Kit v 3 - HS (Illumina). The libraries were indexed with different adaptors, pooled and
94 sequenced in 4 technical replicates.
95 The pipeline used for raw sequence data quality control (QC), estimation of gene transcript
96 levels and differential expression analysis were described in detail previously [11]. The obtained raw
97 reads were aligned to the *Sus scrofa* reference genome (assembly Sscrofa10.2 (GCF_000003025.5)).
98 Differentially expressed genes (DEGs; fold change $\geq |1.2|$; adjusted p-value < 0.05) were determined
99 separately for each breed using Deseq2 software (1.12.4) between pig groups differing in weight of
100 loin muscle. The GO and pathway analyses were performed using Panther and David software [12,
101 13] with *Sus scrofa* reference. The p-value in enrichment tests was determined based on the Mann–
102 Whitney U Test (Wilcoxon Rank-Sum Test) (Panther software)[12], while significantly enriched
103 pathways were detected based on Fisher's exact test (David software)[13].

104 *2.3. miRNAome sequencing and data analysis*

105 microRNA libraries were prepared using the NEBNext Multiplex Small RNA Library Prep Set
106 for Illumina (New England Biolabs) according to the standard protocol. Briefly, the first step was the
107 3′ adaptor ligation, followed by hybridization with the Reverse Transcription Primer and ligation
108 with the 5′ adaptor. The RNA-adaptor ligation products were subjected to reverse transcription.
109 Then, PCR amplification with 12 different indexed primers was performed to allow further
110 multiplexing of the samples. The amplified samples were purified and size-selected on a Novex 6%
111 TBE PAGE gel (Invitrogen). After the overnight elution from the gel, the libraries were precipitated
112 and purified with ethanol. Next, they were subjected to a concentration measurement with a Qubit
113 2.0 Fluorometer (Thermo Fisher Scientific) and a size assessment with a 2200 TapeStation instrument
114 (Agilent). Finally, the obtained libraries were pooled and sequenced by applying 36 cycles on HiScan
115 SQ (Illumina) according to the manufacturer's protocol.
116 The obtained reads were converted to FastQ files, subjected to demultiplexing using bcl2fastq
117 software (Illumina), and quality controlled using FastQC software [14]. The obtained sequences were
118 analysed using UEA sRNA Workbench V3.2 [15]. The first step was 3′ adaptor trimming and filtering
119 according to the following parameters: minimum abundance of at least 6 supporting reads, 17-25 nt
120 of length, low complexity as well as tRNA and rRNA sequences removed. microRNAs were
121 identified by applying the miRCat tool with default parameters for animals, except for minimum
122 abundance (6 reads), minimum length (17 nt), and maximum length (25 nt). The *Sus scrofa* genome
123 (assembly Sscrofa 10.2) and miRBase v21.0 [16,17] were used as references. Detected potentially novel
124 miRNAs were additionally controlled for the presence of other non-coding RNAs using the
125 RNAcentral database [18]. In the last step, the identification of microRNA length and sequence
126 variants – isomiRs – was performed. The analysis was carried out using isomiR-SEA software [19]
127 with the default settings.
128 Differential expression analysis using DESeq2 software [20] was carried out for each breed
129 separately, and significantly expressed miRNAs ($p \leq 0.05$) were chosen for further analyses.
130 miRNAs identified as differentially expressed in the examined samples were analysed using the
131 mirPath v.3 DIANA Tools web application [21] to identify their target genes and biological processes
132 enriched by them. DIANA – TarBase v7.0 with experimentally validated target genes was used as a
133 reference database of target genes, and the KEGG Pathway Database was used as a reference database
134 of biological pathways. The analysis was performed on the basis of human homologues deposited in
135 miRBase (21.0) due to the lack of data for pig microRNAs.

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137 *2.4. Validation of NGS data by qPCR*

138 The validation of RNA-seq results was performed for 6 genes. The exact transcript abundance
139 was measured using real-time PCR analysis. The primers for all genes were designed using Primer
140 4.0 software based on the Ensemble reference genome. cDNA was synthesized from 200 ng of total
141 RNA using a High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Thermo Fisher) according to
142 the protocol. qPCR was performed in 45 cycles on a QuantStudio 7Flex system (Applied Biosystems)
143 with three replicates for each sample (AmpliQ 5x HOT EvaGreen qPCR Mix; Novazym; Poland).
144 *OAZ1* and *RPL27* were used as endogenous controls. The details of the validated genes are presented
145 in Supplementary Table S1. The expression levels were calculated using the delta-delta Ct method
146 [22]. The correlation coefficients between RQ (relative quantity) values and normalized read counts
147 (RNA-seq) were calculated using Pearson's correlation analysis (SAS software).
148 RT-qPCR method was also employed to validate the expression levels of 9 selected microRNAs
149 in all samples (Supplementary Table 1). Reverse transcription reactions were performed using a
150 TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific) according to the
151 manufacturer's protocol. Next, quantitative PCR reactions were run using TaqMan Fast Advanced
152 Master Mix (Thermo Fisher Scientific) and commercially available TaqMan microRNA Advanced
153 Assays (Thermo Fisher Scientific), following the protocol and including Non-Template Control
154 (NTC) for each microRNA assay. The reactions were run in triplicate on a QuantStudio 7 Flex Real-
155 Time PCR System (Thermo Fisher Scientific). The efficiency of amplification reactions for each of the
156 miRNA assays was calculated based on the standard curve method.
157 The NormFinder software was employed to choose an endogenous control that is a microRNA
158 with the most stable expression profile (miR-103a-3p; miR-20a-5p). Finally, relative expression levels
159 of the examined microRNAs were calculated using the $\Delta\Delta$Ct method, including reaction efficiency E
160 [22].

161 **3. Results**

162 *3.1. Transcriptome analysis – differentially expressed genes (DEGs)*

163 According to the RNA sequencing data, on average 17.5 M reads per sample were obtained. On
164 average, 98.3% of all sequences passed QC, of which 75% (12.6 M) mapped to the reference genome.
165 Additionally, 74% of reads were mapped to exons, 18% to intronic regions and 8% to intergenic
166 regions.
167 The RNA-seq approach allowed the identification of 627 and 416 DEGs between the groups with
168 different loin weights (adjusted p-value <0.05; fold change≤ 1.2) for Pietrain and Hampshire,
169 respectively. Next, the comparison of obtained gene sets showed 43 DE genes identified in both
170 breeds (Supplementary Table S2). The up-regulated (30 genes) and down-regulated (13 genes) genes
171 in muscles of pigs with higher muscle mass are presented in Figures 1 and 2, respectively. Gene
172 Ontology analysis showed that the detected genes were involved in the regulation of 'metabolic
173 process' (14 genes - *CYP51; ATGL; TBX2; CEBPD; RLF; JUP; RNF19A*); 'cellular process' (GO:0009987)
174 (10 genes - *RNF19A; UCP3; NAA15; ATGL; PCMT1; JUP*) and 'developmental process' (GO:0032502)
175 (3 genes – *CEBPD; TBX2; SETD2*). According to David software, several of the genes were classified
176 as involved in 'developmental growth' or 'skeletal system development' (*PCOLCE; BBS2; TBX2;*
177 *RBBP6; ATAF5*). The most over-represented DEGs encoded protein classes related to signal
178 transduction, nucleic acid binding and enzyme modulators (Table 2).

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180 **Table 2.** The detected significant Gene Ontology terms based on genes differentially expressed
181 between pigs differing in terms of muscle mass.

GO term	P value/genes	Number of genes detected/ genes		Hp	Pi
Signal transduction	0.034	11		FC	FC
	ENSSSCG00000013901	*IFI30*	IFI30, lysosomal thiol reductase	1.82	1.37
	ENSSSCG00000009668	*CLU*	clusterin	1.92	1.45
	ENSSSCG00000028022	*COL6A2*	collagen type VI alpha 2 chain	1.71	1.36
	ENSSSCG00000022236	*FOLR1*	folate receptor 1 (adult)	2.01	1.53
	ENSSSCG00000022246	*ICOSLG*	inducible T-cell costimulator ligand	1.83	1.39
	ENSSSCG00000011129	*ITIH5*	inter-alpha-trypsin inhibitor heavy chain family member 5	1.67	1.38
	ENSSSCG00000000606	*MGP*	matrix Gla protein	2.04	1.30
	ENSSSCG00000026934	*PIGX*	phosphatidylinositol glycan anchor biosynthesis class X	1.27	1.36
	ENSSSCG00000027466	*PCOLCE*	procollagen C-endopeptidase enhancer	2.23	1.27
	ENSSSCG00000013181	*SERPING1*	serpin family G member 1	2.38	1.27
	ENSSSCG00000014834	*UCP3*	uncoupling protein 3	2.73	1.68
Nucleid acid binding	0.010	9			
	ENSSSCG00000014834	*UCP3*	uncoupling protein 3	2.73	1.68
	ENSSSCG00000007830	*RBBP6*	RB binding protein 6, ubiquitin ligase	2.06	1.25
	ENSSSCG00000027060	*TBX2*	T-box 2	1.73	1.39
	ENSSSCG00000011332	*SETD2*	SET domain containing 2	1.87	1.27
	ENSSSCG00000006276	*CEBPD*	CCAAT/enhancer binding protein delta	2.57	1.38
	ENSSSCG00000027946	*MVP*	major vault protein	1.32	1.53
	ENSSSCG00000003670	*RLF*	rearranged L-myc fusion	1.80	1.25
	ENSSSCG00000026631	*SLC25A6*	solute carrier family 25 Member 6	1.73	1.35
	ENSSSCG00000028035	*ZC3H11A*	zinc finger CCCH-Type Containing 11A	2.73	1.68
Cytoplasm	0.032	5			

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	ENSSSCG00000025417	*BBS2*	Bardet-Biedl syndrome 2	1.37	1.35
	ENSSSCG00000009668	*CLU*	clusterin	1.92	1.45
	ENSSSCG00000017428	*JUP*	junction plakoglobin	1.31	1.39
	ENSSSCG00000004103	*PCMT1*	protein-L-isoaspartate (D-aspartate) O-methyltransferase	1.46	1.26
	ENSSSCG00000006066	*RNF19A*	ring finger protein 19A, RBR E3 ubiquitin protein ligase	2.10	1.31
Enzyme modulator	0.015	4			
	ENSSSCG00000003201	*ATF5*	activating transcription factor 5	1.51	1.54
	ENSSSCG00000013181	*SERPING1*	serpin family G member 1	2.38	1.27
	ENSSSCG00000011129	*ITIH5*	inter-Alpha (Globulin) Inhibitor H5	1.67	1.38
	ENSSSCG00000013074	*RAB3IL1*	RAB3A Interacting Protein Like 1	2.16	1.59
Cytoskeleton	0,021	3			
	ENSSSCG00000025417	*BBS2*	Bardet-Biedl syndrome 2	1.37	1.35
	ENSSSCG00000017428	*JUP*	junction plakoglobin	1.31	1.39
	ENSSSCG00000006066	*RNF19A*	ring finger protein 19A, RBR E3 ubiquitin protein ligase	2.10	1.31
Methyltransferase	0.0066	3			
	ENSSSCG00000011332	*SETD2*	SET domain containing 2	1.87	1.27
	ENSSSCG00000015311	*CYP51*	cytochrome P450, family 51, subfamily A, polypeptide 1	2.27	1.41
	ENSSSCG00000004103	*PCMT1*	protein-L-isoaspartate (D-aspartate) O-methyltransferase	1.46	1.26

182 Hp – Hampshire; Pi – Pietrain; FC – fold change obtained for DEGs between different phenotypic
183 groups in each breed

184 In both breeds, in the pigs with higher muscularity, the most up-regulated *UCP3* gene coding
185 for mitochondrial uncoupling protein 3 was primarily expressed in skeletal muscle and regulating
186 the tissue respiratory processes (fold change 2.73 and 1.68 for Hampshire and Pietrain, respectively).
187 On the other hand, genes showing the greatest significant expression decrease in the pigs with higher
188 loin weight (compared to the animals with lower muscle mass) were *MED13* (encoding Mediator
189 Complex Subunit 13; FC= -2.40 and -1.39 for H and P pigs) and *CYP51* (encoding Cytochrome P450
190 Family 51 Subfamily A Member 1; FC=-2.26 and -1.41 for H and P pigs) genes.

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191 The RNA-seq experiment was submitted to the NCBI Gene Expression Omnibus (GEO)
192 functional genomics data and assigned the GEO accession number GSE107207.

193 *3.2. miRNAome analysis – differentially expressed miRNA*

194 As a result of the miRNAome sequencing, between 2.5 M and 7.6 M raw sequences in individual
195 samples were identified. After the filtering, between 2.4 M (94.6%) and 5.8 M (77%) sequences were
196 further mapped to the reference genome.
197 miRNAome sequencing allowed the detection of 196 known and 149 potentially new
198 microRNAs in both breeds. In the Pietrain samples, 160 known and 59 potentially novel miRNAs
199 were identified, while for the Hampshire pigs, 192 known and 138 potentially new miRNAs were
200 detected. The comparison of miRNA profiles between pig groups with different loin weights showed
201 differential expression of 57 microRNAs for Hampshire pigs (24 up-regulated and 34 down-
202 regulated; Supplementary Figures S1 and S2) and 34 miRNAs for Pietrain pigs (28 up-regulated and
203 6 down-regulated; Supplementary Figures S3 and S4). The most significant ($p<0.01$) miRNAs detected
204 in Pietrain and Hampshire pigs are shown in Figures 3 and 4. Furthermore, 18 common microRNAs
205 with differential expression were identified for both tested breeds (miR-499a-5p; miR-206; miR-146a-
206 5p; miR-133a-3p; miR-378b; miR-128-3p; miR-378b-3p; miR-10b; miR-451a; miR-125b; miR-30a-5p;
207 let-7f-5p; let-7i-5p; let-7g-5p; miR-7-5p; miR-26a-5p; miR-185-5p; miR-126-3p). This finding may
208 indicate the existence of muscle growth with the regulating pathways common for both investigated
209 high-muscularity pig breeds.
210 Eighteen DE microRNAs detected in both breeds were further subjected to Gene Ontology and
211 KEGG Pathway analyses (mirPath v.3). According to an experimentally validated target gene
212 database (Tarbase v7.0), the most over-represented GO processes were identified, such as cell cycle,
213 fatty acid biosynthesis, regulation of actin cytoskeleton as well as lysine degradation and ubiquitin
214 mediated proteolysis (Table 3). All identified GO biological processes related to 18 DE microRNAs
215 are shown in Figure 5. The GO terms in each breed and taking into account the direction of expression
216 change is presented in Supplementary Files 3-6.
217
218
219

220 **Table 3.** The detected significant Gene Ontology terms based on miRNAs differentially expressed
221 between pigs differing in terms of muscle mass.

GO	Detected miRNA	N	Most interesting genes related with GO term	
Cell cycle (hsa04110)	hsa-let-7i-5p	32	*CDK4; CDK2; CDK6; TP53; RBL2; ESPL1; ORC2*	p value 1.02676902709e-09
	hsa-let-7g-5p	33	*CDK4; CDK2; CDK6; TP53; RBL2; ESPL1; ORC2*	
	hsa-miR-30a-5p	24	*PCNA; SMAD2; TP53; MYC; YWHAE*	
	hsa-let-7f-5p	35	*ESPL1; CCNB2; RBL2; SMC1A; CDK4; TP53; ATR*	
	hsa-miR-499a-5p	7	*YWHAG; E2F5; SKP2; MYC; CDC27; PRKDC; MDM2*	
	hsa-miR-146a-5p	12	*GSK3B; CCNB1; CCNA2; SMAD4; RBL1; CDC23; PRKDC; MDM2; ABL1*	
	hsa-miR-378a-3p	20	*CCNB1; YWHAG; CCND2; MCM4; CDK1; BUB3; MDM2*	
	hsa-miR-10b-5p	13	*YWHALE; CDK2; CCND2; CUL1; TP53; CDC27*	
	hsa-miR-7-5p	20	*YWHAH; YWHAE; CCNB1; E2F2; CDK2; ATM;*	
	hsa-miR-26a-5p	32	*CDC6; CCNB1; YWHAE; YWHAG; YWHAZ; SMAD4; WEE1; EP300*	
	hsa-miR-185-5p	16	*CDK4; YWHAE; YWHAG; YWHAB; YWHAQ; ORC4;*	
	hsa-miR-126-3p	4	*E2F1; E2F3; CCNE2; CCNE1*	
	hsa-miR-125b-5p	19	*CDC6; E2F2; E2F3; ATR; TP53; CUL1; ORC5*	
	hsa-miR-206	9	*CDK4; CCND2; CDC7; WEE1; MCM7; CDC25C*	

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Fatty acid biosynthesis (hsa00061)	hsa-miR-30a-5p	4	*FASN; ACSL1; ACSL3; ACSL4*	6.03836341862e-09
	hsa-miR-26a-5p	4	*FASN; ACSL1; ACSL3; ACSL4*	
	hsa-miR-378a-3p	2	*ACSL4; ACACA*	
	hsa-miR-7-5p	4	*FASN; OXSM; ACACA; ACSL4*	
	hsa-let-7i-5p	1	*MCAT*	
	hsa-let-7g-5p	1	*MCAT*	
	hsa-let-7f-5p	1	*MCAT*	
	hsa-miR-125b-5p	1	*FASN*	
Regulation of actin cytoskeleto n (hsa04810)	hsa-let-7i-5p	31	*PPP1CA; PFN1; BRAF; ACTB; SSH2; CRK; GNA13; FN1; RAC1; VAV3; MAPK1; FGFR1*	0.021867
	hsa-let-7g-5p	31	*PPP1CA; PFN1; BRAF; ACTB; SSH2; CRK; GNA13; FN1; RAC1; VAV3; MAPK1; FGFR1*	
	hsa-miR-125b-5p	27	*SSH2; EZR; NRAS; CRKL; CRK; PAK2; PXN; MYL9; FGFR2; FGFR1; MYH9*	
	hsa-let-7f-5p	32	*PPP1CA; PFN1; BRAF; ACTB; SSH2; CRK; GNA13; FN1; RAC1; VAV3; MAPK1; FGFR1*	
	hsa-miR-378a-3p	17	*ACTB; SSH2; ITGA9; CRKL; GNA13; PFN2; PAK4; MAPK1; MYLK*	
	hsa-miR-146a-5p	9	*BRK1; ROCK1; WASL; EDFR; ACTN1; ITGB2; WASF2*	
	hsa-miR-7-5p	35	*ACTB; ACTN2; NRAS; APC; RRAS2; FN1; FGF1; ACTN4; MAPK1; PIK3CB; EGFR*	
	hsa-miR-185-5p	14	*PIP5K1C; FN1; MYLK; ENAH; CDC42; ITGB5; RHOA; EGFR; PPP1CC*	
	hsa-miR-378b	4	*SSH2; ITGB1; ROCK2; MSN*	
	hsa-miR-26a-5p	32	*SSH2; EZR; NRAS; CRK; ITGA8; PAK1; ITGB3; ITGA6*	
	hsa-miR-30a-5p	29	*ARPC5; WASL; NRAS; SSH1; GNA13; EGFR; FN1; RAC1; PIK3CA; PPP1CB*	
	hsa-miR-10b-5p	6	*CRK; ARPC5; ACTG1; TIAMI; PIK3CD; DIAPH2*	
	hsa-miR-126-3p	8	*PIK3CA; PIK3CD; ITGA6; MAPK1; KRAS; CRK; PIK3R2*	
	hsa-miR-451a	3	*BRAF; WASL; PIK3CA*	
	hsa-miR-206	2	*EGFR; FN1*	
	hsa-miR-499a-5p	2	*CFL2; FGF2*	
Lysine degradation (hsa00310)	hsa-let-7i-5p	12	*WHC1L1; SETD1B; PLOD2; ASHIL; SETD1A; KMT2B*	7.85414955559e-07
	hsa-let-7g-5p	11	*PLOD2; SETD1B; ASH1L; DOT1L; KMT2D; KMT2E*	
	hsa-miR-125b-5p	7	*KMT2D; WHSC1; KMT2C; KMT2B; SUV39H1; SUV420H2*	
	hsa-let-7f-5p	12	*ALDH7A1; SETD1B; KMT2D; DOT1L; SETD1A; KMT2B; KMT2E*	
	hsa-miR-7-5p	15	*ALDH3A2; SETD1B; PLOD2; PLOD1; SETD2; NSD1; OGDH;*	
	hsa-miR-378a-3p	4	*SETD7; ASH1L; KMT2D; SUV420H1*	
	hsa-miR-30a-5p	12	*SETD7; PLOD2; OGDH; NSD1; SETD2; ALDH2*	
	hsa-miR-185-5p	3	*ASHIL; KMT2D; WHSC1*	
	hsa-miR-10b-5p	4	*SETD7; SUV420H1; WHSC1L1; ALDH3A2*	
	hsa-miR-26a-5p	9	*SETD2; NSD1; ACAT2; SETD8; SETDB1; KMT2D; KMT2C; KMT2A; ALDH9A1*	
	hsa-miR-146a-5p	2	*NSD1; KMT2C*	
Ubiqui tin mediat	hsa-let-7i-5p	29	*BCBL; MAP3KI; UBE3B; BTRC; HUWEI; SKP2; NEDD4L; MID1U; BOX5*	9.766 01497

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hsa-let-7g-5p	30	*UBE3B; CBL; CUL2; NEDD4L; MAP3K1; MID1; BIRC3; UBOX5; SKP2*
hsa-miR-30a-5p	42	*UBE3B; CUL2; NEDD4L; MAP3K1; MID1; BIRC3; UBOX5; SKP2; UBA6; SEA1*
hsa-let-7f-5p	29	*UBE2Z; CBL; UBE2R2; UBR5; STUB1; XIAP; FBXW7; BIRC6*
hsa-miR-7-5p	23	*UBE2Z; CBL; UBE2R2; UBR5; STUB1; XIAP; FBXW7; BIRC6*
hsa-miR-26a-5p	23	*WWP2; SMURF2; HUWE1; SKP1; BRCA1; CDC23; MDM2;*
hsa-miR-378a-3p	9	*UBE2Z; CBL; BTRC; FBXW7; HUWE1; PML; RBX1; MDM2*
hsa-miR-206	1	*BRCA1*
hsa-miR-146a-5p	6	*ITCH; MID1; BRCA1; TRAF6; CDC23; MDM2*
hsa-miR-10b-5p	11	*UBE2Z; UBE3B; CUL1; BRCA1; NEDD4; CDC27; MDM2*
hsa-miR-185-5p	12	*UBE2Z; UBR5; HUWE1; BIRC6; MDM2; TRIP12; UBE4A*
hsa-miR-125b-5p	10	*HUWE1; CUL1; UBA6; BIRC6; SAE1; CDC27; XIAP*
hsa-miR-499a-5p	4	*SKP2; CDC27; MDM2; KLHL9*
hsa-miR-126-3p	2	*UBE2K; FBXW11*
hsa-miR-378b	1	*RBX1*

222

223 The miRNA experiment was submitted to the NCBI GEO database and assigned the GEO
224 accession number GSE109215.

225 *3.3. Integrated analysis of transcriptome and miRNAome*

226 The pathway analysis based on 43 DEGs allowed us to identify three pathways in which two
227 genes coding for collagens (*COL6A1* and *COL6A2*) were involved: the PI3K-Akt signalling (ssc04151),
228 Focal adhesion (ssc04510) and ECM-receptor interaction (ssc04512) pathways. Furthermore, two
229 genes (*CYP51* - Cytochrome P450 51A; *PIGX* - Phosphatidylinositol Glycan Anchor Biosynthesis
230 Class X) belonging to the metabolic pathway (ssc01100) were identified. The KEGG pathway analysis
231 based on miRNA results indicated that the most significant pathway was the Hippo signalling
232 pathway, which is also connected with the TGF-beta signalling pathway and controls organ size via
233 the regulation of ubiquitin-mediated proteolysis, cell proliferation and apoptosis (Figure 6). The most
234 interesting target genes involved in identified pathways (FoxO signalling pathway, TGF-beta
235 signalling pathway, signalling pathways regulating pluripotency of stem cells and PI3K-Akt
236 signalling pathway) were *SOX2, SIRT1, KLF4, PAX6* (Figure 7) and other genes belonging to the
237 transforming growth factor beta superfamily (Table 4).

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238 **Table 4.** The pathways and genes regulated by identified miRNAs (N-number of genes regulated in each pathway).

Pathway	miRNA involved in pathway regulation	N	Target genes	P value
Hippo signaling pathway (hsa04390)	hsa-miR-499a-5p; hsa-miR-30a-5p, hsa-miR-7-5p; hsa-let-7i-5p; hsa-let-7g-5p; hsa-miR-125b-5p; hsa-let-7f-5p; hsa-miR-26a-5p; hsa-miR-185-5p; hsa-miR-146a-5p;hsa-miR-10b-5p; hsa-miR-378a-3p; hsa-miR-451a; hsa-miR-126-3p; hsa-miR-206; hsa-miR-378b	87	*PPP1CA; ACTB; TGFBR1; BMP5; BMPR1B; WNT5A; YWHAE; WNT10B; WNT3A; WNT8B; BMP2; YWHAB;; YWHAQ; MOB1B; SMAD4; BMP4; SOX2; BMPR2*	6.03836341862e-09
FoxO signaling pathway (hsa04068)	hsa-let-7i-5p; hsa-let-7g-5p; hsa-let-7f-5p; hsa-miR-206; hsa-miR-378a-3p; hsa-miR-30a-5p; hsa-miR-7-5p; hsa-miR-185-5p; hsa-miR-26a-5p; hsa-miR-126-3p; hsa-miR-499a-5p; hsa-miR-146a-5p; hsa-miR-451a; hsa-miR-125b-5p; hsa-miR-10b-5p	88	*BRAF; NRAS; KRAS; TGFBR1; TGFBR2; PIK3R2; FBX032; IGFR; SIRT1; IRS2; FOXO6; FOXO3; USP7;*	2.15944221889e-08
TGF-beta signaling pathway (hsa04350)	hsa-miR-10b-5p; hsa-let-7i-5p; hsa-let-7g-5p; hsa-miR-30a-5p; hsa-let-7f-5p; hsa-miR-26a-5p;hsa-miR-378a-3p; hsa-miR-499a-5p; hsa-miR-451a; hsa-miR-7-5p; hsa-miR-126-3p, hsa-miR-146a-5p; hsa-miR-125b-5p; hsa-miR-185-5p; hsa-miR-378b; hsa-miR-206	50	*FST; TGFBR1; ID2; ID4; ROCK1; SMAD2; SMAD3; SMAD4; SMAD7; SP1; BMP4; BMP2; BMP5; BMPR1B; BMPR2; MAPK1;*	1.35796585427e-06
PI3K-Akt signaling pathway (hsa04151)	hsa-miR-30a-5p; hsa-miR-7-5p; hsa-let-7i-5p; hsa-miR-10b-5p; hsa-let-7g-5p; hsa-let-7f-5p; hsa-miR-125b-5p;hsa-miR-185-5p; hsa-miR-206; hsa-miR-378a-3p; hsa-miR-26a-5p; hsa-miR-499a-5p; hsa-miR-146a-5p; hsa-miR-126-3p;hsa-miR-451a;hsa-miR-378b	167	*TLR2; PRLR; MET; ITGB1; ATF2; CDK2; GNG5; GNG11; GNG12; CDK2; STK11; CK6; IGF1R; EGFR; TP53; PIK3CD; PIK3R3; FGF9; ITGA5; FGF1*	0.0162065002097
Signaling pathways regulating pluripotency of stem cells (hsa04550)	hsa-miR-125b-5p; hsa-miR-30a-5p;hsa-miR-378a-3p; hsa-miR-10b-5p; hsa-let-7i-5p; hsa-let-7g-5p; hsa-let-7f-5p;hsa-miR-7-5p;hsa-miR-26a-5p; hsa-miR-146a-5p; hsa-miR-185-5p; hsa-miR-126-3p; hsa-miR-499a-5p; hsa-miR-451a	86	*SOX2; KLF4; PAX6; PIK3R2; FZD3; MAPK13; KRAS; SMAD6; NRAS; IGF1R; TBX3; LIF; MYC; BPO2; FGF2; LIFR; CTNNB1; PIK3CA; SMP4; BMPR2; JAKI*	1.27589892447e-06

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240 Moreover, the analysis of miRNA–target gene interactions was performed with the use of the DIANA-miRextra v2.0 tool to identify functional microRNAs potentially responsible for changes in gene expression identified in the present study as DEGs. mRNA and microRNA differential expression analysis results were simultaneously analysed, and important miRNA regulators were identified based on functional analysis of their target genes. The obtained results confirmed the significant association of 5 detected microRNAs and 9 DEGs (Table 5). Two genes were identified as the most frequently regulated by detected miRNAs, namely, *CYP51A1*, which is related to cholesterol metabolism (hsa-miR-155-5p; hsa-miR-30c-5p; hsa-miR-199b-5p), as well as the *RNF19A* gene, which is associated with ubiquitination (hsa-miR-155-5p; hsa-miR-133a-3p; hsa-miR-126-5p) (Table 5).

249 **Table 5.** The miRNA-mRNA interactions identified between differentially expressed miRNAs and genes based on the DIANA-miRextra v2.0 tool

Hampshire		Pietrain	
miRNA	*DEGs*	miRNA	*DEGs*
hsa-miR-155-5p	*RNF19A; FEM1B; ATL2; CYP51A1; XPO1;*	hsa-miR-199b-5p	*CYP51A1*
hsa-miR-30c-5p	*SETD2; ATL2; PIGX; RBB6P; CYP51A1; XPO1;*	hsa-miR-126-5p	*RNF19A1*
hsa-miR-133a-3p	*XPO1; RNF19A; PNPLA2*		

251 *3.4. Validation of the obtained results*

252 The estimation of gene expression using real-time PCR confirmed the direction of transcript level changes between analysed biological groups detected using the RNA-seq approach (FC profiles values). The Pearson correlation calculated between RQ values and normalized read counts (RNA-seq) showed high significant correlation coefficients (from 0.928 to 0.643) for most of the analysed genes (Table 6). For the *TBX2* and *XPO1* genes, the correlation was not significant, but the FC values obtained by these two methods were similar. The validation of miRNA showed a high and significant correlation between NGS and qPCR results for most of the analysed miRNAs (from 0.888 to 0.635) (Table 7). For two miRNAs, miR-7-5p and miR-125b-5p, the obtained correlation coefficients were not significant (0.402 and 0.232, respectively).

261 **Table 6.** The validation results obtained between the correlation of RNA-seq and qPCR data for differentially expressed genes

Gene	Accession number	Hampshire		Pietrain		R
		FC RNA-seq	FC RQ	FC RNA-seq	FC RQ	
MGP	ENSSSCG00000000606	2.45	2.07	1.63	1.80	0.928***
BBS2	ENSSSCG00000025417	-2.25	-1.76	-1.53	-1.03	0.646***
PCOLCE	ENSSSCG00000027466	3.35	2.79	1.57	1.10	0.886***
COL6A1	ENSSSCG00000022506	2.20	1.40	1.97	1.70	0.643**
TBX2	ENSSSCG00000027060	2.05	1.91	2.21	1.09	0.360[ns]
XPO1	ENSSSCG00000028228	-1.71	-0.62	-1.50	-1.80	0.280[ns]

263 R- correlation coefficient; FC – fold change values between groups with high and low meat content in carcasses in each breed; RQ – relative quantity, Pearson correlation coefficient with p value (*** - p<0.000; **-p<0.01; ns – not significant).

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267 **Table 7.** The correlation coefficient of RNA-seq and qPCR data obtained for miRNAs

miRNA	R
let-7g-5p	0.738**
miR- 126-3p	0.794**
miR-181b-5p	0.658*
miR-30a-5p	0.888***
miR-378a-3p	0.635*
miR-451a	0.756**
miR-499a-5p	0.878***
miR-7-5p	0.402[ns]
miR-125b-5p	0.232[ns]

268 R- correlation coefficient; FC – fold change values between groups with high and low meat content in
269 carcasses in each breed; RQ – relative quantity, Pearson correlation coefficient with p value (*** -
270 p<0.0001; **-p<0.01; *-p<0.05; ns – not significant).

271 **4. Discussion**

272 Since the beginning of domestication, selection in pigs has been focused on improving the lean
273 meat content in carcasses. Currently, this direction of selection is most evident in breeds constituting
274 a paternal component in breeding (Duroc, Pietrain and Hampshire pigs), which are used to improve
275 the meat quantity of cross-breed pig lines. Furthermore, even in one breed, a significant variation in
276 the meatiness level can be observed. In the present study, a comprehensive analysis of genes and
277 microRNA profiles in porcine muscle tissue was performed to identify the genetic background of
278 meat content in carcasses. Moreover, NGS analyses were applied to compare global genes and
279 miRNA expression profiles between groups differing in weight of loin muscle in each breed. Such an
280 approach allowed us to avoid a potential breed-specific effect on transcript abundance and to detect
281 differential expression of genes related to the trait of interest.
282 The global gene expression profile in a cell or tissue is a result of the transcription rate as well as
283 other post-transcriptional modifications, including mRNA silencing. One of the main gene's
284 expression regulators is microRNA molecules, which predominantly promote transcript degradation
285 [23]. Commonly, miRNAs regulate transcript stability via binding to the 3'UTR of their targeted genes
286 with the RISC multiprotein complex (RNA-induced silencing complex) [24]. microRNAs can control
287 transcript silencing by involvement in transcriptional inhibition through microRNA-mediated
288 chromatin reorganization, mRNA destabilization by decay or cleavage and regulation of proteins
289 belonging to transcription complexes [25, 26].
290 To date, efforts have been made to identify regulatory mechanisms related to muscle growth
291 and development during ontogenesis in pigs. Using a pathway-focused oligo microarray, Li et al.
292 [27] compared the expression profile of genes involved in muscle growth and fatty acid biosynthesis
293 in muscle tissue collected from two pig breeds at six growth stages. Among the genes significantly
294 deregulated during growth periods, the authors detected the *UCP3* gene (uncoupling protein 3),
295 whose expression was regulated by 30 other identified genes. The *UCP3* gene encodes mitochondrial
296 anion carrier protein, and together with *UCP2*, it is essential for body metabolism regulation [28].
297 Both genes are mainly expressed in muscle tissue and control mitochondrial fatty acid transport and
298 glucose metabolism [29]. In our research, we confirmed the differential expression of the *UCP3* gene
299 between pigs with varied muscling in both breeds. Furthermore, a miRNA–mRNA target analysis
300 showed an interaction between the *UCP3* gene and identified in Hampshire pigs miR-30c-5p, which
301 was established to regulate myoblast differentiation [30]. microRNAs belonging to the miR-30 family
302 also affect the activity of miR-206 – the main modulator of skeletal muscle development [31,32].
303 Muscle-specific miR-206 (myomiR) was detected in the present study as differentially expressed in
304 Pietrain and Hampshire pigs differing in muscle weight. This observation confirmed that miR-206
305 plays an important role in muscle growth and development, regardless of breed tested.
306 Sheng et al. [33] performed whole miRNAome profiling in pig breeds with high lean meat
307 percentage (Large White) and low body weight (Min Pigs) at several postnatal stages and showed

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308 that the one of the most abundant myomiRs was miR-206. Interesting results were reported by Cai et
309 al. [34], who confirmed that the microRNA profile in skeletal muscle was significantly altered by
310 castration. The authors identified seven differentially expressed miRNAs between intact and
311 castrated pigs: miR-206, let-7c and let-7a (down-regulated) and miR-1, miR-133a, miR-26a, and miR-
312 133b (up-regulated). Cai et al. [34] indicated the key impact of castration on miRNA expression,
313 which can explain the main mechanism underlying variations in muscle growth in intact and
314 castrated pigs. Our study allowed us to detect three of seven previously described miRNAs (miR-
315 206; miR-26a; miR-133a). Moreover, we detected the expression of three microRNAs (let7i; let-7g; let-
316 7f) belonging to the let-7 family, which are also known as important developmental regulators.
317 Previous studies performed by Huang et al. [35] and Yan et al. [36] showed that skeletal muscle
318 hypertrophy can be controlled by the IGF-1–Akt pathway and myostatin signalling pathway. It
319 results from the regulation of *IGF-1* or *IGFR* expression by miR-133a/b and miR-206. Furthermore,
320 during exercise-induced muscle response, IGF-1 signalling can be modified by miR-126, which also
321 impacts *MyoD* and *Myf5* genes strongly related to myogenesis [37]. In humans, several muscle-
322 specific miRNAs, including miR-133a/b and miR-206, are associated with muscular dystrophies
323 (Duchenne and Becker) and are called dystromirs. Their serum expression profile can be used as a
324 biomarker of such muscle disorder [38]. Apart from miR-206 and miR-133a, our results showed
325 significant differences in miR-126-3p expression and significant changes in the expression of its target
326 gene, *RNF19A* (Ring Finger Protein 19A). It has been established that the *RNF19A* gene mainly plays
327 a role in neurons causing amyotrophic lateral sclerosis or Parkinson's disease. On the other hand, it
328 was proposed as one of interesting genes related to muscle response during stabilized weight loss
329 [39].
330 Another gene with modified transcript levels dependent on loin mass was the *SETD2* gene,
331 whose expression is regulated by miR-30c-5p. The *SETD2* gene, which encodes a histone
332 methylotransferase, is involved in the regulation of transcription processes via histone methylation.
333 Recent studies have shown that the *SETD2* gene is involved in epigenetic mechanisms regulating
334 myoblast proliferation and differentiation [40]. Furthermore, using the CRISPR/CAS9 system, the
335 authors indicated that silencing the *SETD2* gene resulted in a decrease in the expression of major
336 regulators of the cell cycle, repression of myogenin – MyoG – transcription and up-regulation of the
337 cyclin-dependent kinase inhibitor p21. The arrest of the cell cycle caused by the silencing of the
338 *SETD2* gene demonstrated that this gene is critical during myoblast proliferation and differentiation
339 [40]. In our study, a significant up-regulation of the *SETD2* gene in pigs characterized by higher
340 muscle mass in carcasses was observed. These results support previous findings and indicate a strong
341 relationship between the *SETD2* gene and myogenesis in pigs.
342 The comparison of the global miRNA profile in muscle tissue in the pigs differing in terms of
343 muscularity allowed for the detection of some pathways regulated by identified differentially
344 expressed miRNAs. The most significantly enriched pathways were the Hippo signalling pathway,
345 FoxO signalling pathway and signalling pathways regulating pluripotency of stem cells. Numerous
346 studies have shown that the Hippo signalling pathway plays a key role in muscle cell proliferation,
347 differentiation and apoptosis and, as a result, controls myogenesis processes [36, 41-44]. The Hippo
348 signal transduction network can regulate muscle mass and organ size via the activation of cascades
349 of serine/threonine kinases [45, 46]. Moreover, Gnimassou et al. [46] suggested that this pathway can
350 contribute to changes and adaptation of muscle mass after exercise. microRNAs identified in the
351 present study can be upstream regulators of the Hippo pathway and, as described previously, are
352 considered to be myomiRs closely related to muscle growth. Our results showed that several miRNAs
353 (miR-30a-5p; miR-206; miR-26a-5p; miR-499a-5p; miR-146a-5p) also regulated the FoxO, TGF-beta,
354 PI3K-Akt signalling pathways and signalling pathways regulating pluripotency of stem cells. The
355 identified miRNAs regulate genes coding for proteins belonging to the 14-3-3 family (*YWHAE;*
356 *YWHAB; YWHAQ*), which are considered important versatile regulators of the cell cycle, metabolism,
357 cell signalling and apoptosis [47; 48]. It was established that 14-3-3 proteins act through regulation of
358 FOXO transcription factors – especially FoXO3, whose significant role in controlling modifications of
359 muscle mass (hypertrophy or atrophy) was confirmed [49]. Furthermore, the identified microRNAs

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control the transcript abundance of genes regulating different aspects of development: TGF-beta proteins (*TGFBR1; BMP2; BMP4BMP5; BMPR1B; BMPR2*) and genes recognized as pluripotency markers (*SOX2; KLF4).* The deregulation of the detected DE miRNAs and genes can provide newinformation about processes related to the growth and development of muscle tissue in pigs.

The *PCOLCE* gene (Procollagen C-Endopeptidase Enhancer), which is involved in muscle growth disorders in humans, was detected in the set of DEGs in Pietrain and Hampshire breeds. This gene encodes an enzyme responsible for the transformation of procollagens to collagens and is associated with the deregulation of extracellular matrix (ECM) functions and, as a result, with the appearance of late-onset muscle fibrosis and Oculopharyngeal Muscular [50]. The extracellular matrix plays a key role during signal transduction, and any disorders of this function can lead to myopathies. However, the exact mechanism of the association between impaired ECM matrix function and muscle growth remains unknown. The differential expression of the *PCOLCE* gene in muscle tissue diverting in mass suggests that this gene can contribute to porcine muscle growth via modification of the ECM role. In both breeds, the differential expression of two genes coding for collagens were detected, namely, *COL6A1* and *COL6A2.* Both *COL6A2* and its paralog *COL6A1* contribute to the organization of matrix components by binding several extracellular matrix proteins and are related to the occurrence of muscle dystrophy and myopathy (Bethlem Myopathy 1 and Ullrich Congenital Muscular Dystrophy 1) [51, 52]. These findings also support the hypothesis that ECM matrix remodelling is associated with the variation of muscle tissue growth and development.

Farther, the *TBX2* gene coding for the T-box transcription factor was identified in our study as up-regulated in pigs with higher muscle mass. In humans, it was confirmed that *TBX2* is responsible for developing tissue sarcomas in which this gene is overexpressed. Zhu et al. [53] indicated that *TBX2* regulates the skeletal muscle cell cycle via an interaction with the myogenic transcription factor MyoD and the cell cycle regulators p21 and p14. The authors suggested that in sarcomas, *TBX2* promoted cell proliferation, and inhibition of its expression led to the repression of tumour growth [53, 54].

5. Conclusion

The comprehensive analysis of the transcriptome and miRNAome profile in porcine muscle tissue differing in terms of muscle mass allowed us to identify differentially expressed genes and miRNAs potentially related to muscle growth and development. We detected microRNAs characteristic of muscle tissue, such as microRNAs belonging to the miR-30 family, miR-206, miR-26a and miR-133a, which have been established as the main modulators of skeletal muscle development. The identification of DE genes and microRNAs enabled us to pinpoint interesting molecular pathways and biological processes essential in controlling muscle growth and development in the pig. The close relationship of some identified in the present study genes and miRNAs with muscle disorders in humans can confirm their significant role in the myogenesis process in pigs. The presented molecular networks show new possibilities for searching candidate genes related to the process of developing muscle tissue and as a result with important production traits in pigs.

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