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Transcriptional profiling during foetal skeletal muscle development of Piau and Yorkshire–Landrace cross-bred pigs

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Summary

Skeletal muscle development is a complex process involving the coordinated expression of thousands of genes. The aim of this study was to identify differentially expressed genes in longissimus dorsi (LD) muscle of pigs at 40 and 70 days (d) of gestation (developmental stages encompassing primary and secondary fibre formation) in Yorkshire–Landrace (YL) cross-bred pigs and Piau pigs (a naturalized Brazilian breed), which are two breed types that differ in muscularity. Foetuses were obtained from gilts at each gestational age (n = 3 YL; n = 4 Piau), and transcriptional profiling was performed using the Pigoligoarray microarray containing 20 400 oligonucleotides. A total of 486 oligonucleotides were differentially expressed (fold change (FC) ≥ 1.5; false discovery rate (FDR) ≤ 0.05) between 40 and 70 d gestation in either YL or Piau pigs, and a total of 1300 oligonucleotides were differentially expressed (FC ≥ 1.5; FDR ≤ 0.05) between YL and Piau pigs at either age. Gene ontology annotation and pathway analyses determined functional classifications for differentially expressed genes and revealed breed type–specific developmental expression patterns. Thirteen genes were selected for confirmation by qRT-PCR analyses, and expression patterns for most of these genes were confirmed, providing further insight into the roles of these genes in pig muscle development. This study revealed both developmental and breed type–specific patterns of gene expression in foetal pig skeletal muscle, including genes not previously associated with myogenesis. This information will contribute to future pig genetic improvement efforts.

Keywords foetal skeletal muscle, pig, transcriptional profiling.

Introduction

The most abundant tissue in animals is skeletal muscle, which typically accounts for 40–65% of the carcass weight in meat animal species. Development, growth and function of skeletal muscle are dynamic processes critical to animal survival and involve the coordinated expression of thousands of genes. Development of skeletal muscle fibres during foetal growth in mammals takes place in two waves, known as primary and secondary fibre formation (Wigmore & Evans 2002). During each wave, myoblasts proliferate and fuse to form new muscle fibres. Primary fibres are formed *de novo*, whereas secondary fibres form around a primary fibre. In pigs, this process takes place at approximately 30-60 days (d) of gestation and 54–90 d of gestation for primary and secondary fibres, respectively (Wigmore & Stickland 1983).

Several previous studies have evaluated gene expression patterns during pig foetal or embryonic development (Yelich *et al.* 1997; Wilson *et al.* 2000; Wesolowski *et al.* 2004). In addition, several studies have examined transcriptional profiles during various stages of pig skeletal muscle development in different breeds (Zhao *et al.* 2003; Lin & Hsu 2005; te Pas *et al.* 2005; Cagnazzo *et al.* 2006; Muráni *et al.* 2007; Tang *et al.* 2007; Li *et al.* 2008; Lobjois *et al.* 2008). It appears from these studies that there are breed-specific patterns of gene expression in developing pig skeletal muscle. In addition, as transcriptional profiling resources for exploring global gene expression patterns in the pig improve, it will be possible to gain further insight into the genes that are expressed during primary and secondary myofibre formation in pigs.

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Piau is a naturalized Brazilian breed, and genetic diversity exists within its current population (Sollero *et al.* 2009). Peixoto *et al.* (2006) have described this breed previously. The high fat content of Piau carcasses can be desirable in cross-breeding programs for improving meat quality traits such as marbling. Historically, this breed was used for its fat and meat characteristics in the 1940s and 1950s in some genetic improvement programmes (Vianna 1956). Piau pigs have also recently been used for the development of a resource population to identify quantitative trait loci for growth, carcass merit, meat quality, and reproductive traits (Peixoto *et al.* 2006; Silva *et al.* 2008; Paixão *et al.* 2008). However, investigation of Piau pigs at the transcriptome level has not been explored.

In this study, we used a whole-genome microarray to identify differentially expressed genes in longissimus dorsi (LD) muscle of Piau and Yorkshire–Landrace (YL) cross-bred pigs at 40 and 70 d of gestation. The two developmental ages examined encompass the two waves of primary and secondary myofibre formation in pigs (Wigmore & Stickland 1983), and the two breed types allowed comparison of breeds with distinctly different muscularity. Differentially expressed genes were functionally characterized, and selected genes were confirmed by quantitative real-time PCR analyses, in order to increase our understanding of myogenesis in pigs.

Materials and methods

Animals and tissue sampling

Animal handling was done in accordance with regulations approved by the institutional animal welfare and ethics/protection commission of the Federal University of Viçosa (UFV; DVT-UFV 02/2008) or by the Michigan State University (MSU) Institutional Animal Care and Use Committee (11/04-141-00). At the UFV Pig Breeding Farm, pregnant Piau gilts at either 40 d of gestation ($n = 4$) or 70 d of gestation ($n = 4$) were aborted using the following protocol: intramuscular injections of 1 mL Prelobam® (PGAF-α)-plus 1 ml oestrogen, followed 12 h later by 2 ml Orastina® (Ocitocine). Longissimus dorsi muscle samples were isolated from foetuses and placed in sterile tubes containing RNA*later*® (Qiagen). Samples were stored at 4 °C overnight and at −80 °C prior to RNA isolation. Pig foetuses were also collected from Yorkshire × Landrace (YL) cross-bred gilts at 40 ($n = 3$) and 70 ($n = 3$) d of gestation by slaughtering gilts in the federally inspected MSU Meats Laboratory. The YL pigs represented a prevalent white cross-bred pig used for breeding in North America. Samples of LD muscle were obtained from foetuses, and then flash frozen in liquid nitrogen and stored at −80 °C.

Piau gilts were aborted to preserve the limited number of animals available for breeding at UFV, whereas abortion was not possible at MSU. To confirm that the method for obtaining foetuses did not affect foetal LD muscle gene expression for breed-type comparisons, a Piau gilt was slaughtered at 40 d of gestation. Expression patterns of three genes subsequently observed to be differentially expressed between the Piau and YL breed types at 40 d of gestation were evaluated by quantitative real-time reverse transcription PCR (qRT-PCR) in foetuses from the aborted and slaughtered gilts. The delta cycles to threshold values of the genes *nebulin-related anchoring protein* (*NRAP*), *ornithine decarboxylase 1* (*ODC1*) and *TIMP metallopeptidase inhibitor 3* (*TIMP3*) for foetuses from the slaughtered gilt were comparable to those of the foetuses from the aborted gilts. (See real-time quantitative PCR methods and results for further details regarding *NRAP*, *ODC1* and *TIMP3*) In addition, foetal LD samples from six foetuses from the slaughtered Piau gilt were divided into two subsamples, with one subsample preserved in RNA*later*® and the other subsample preserved in liquid nitrogen. These duplicate samples were evaluated by qRT-PCR for *NRAP* and *ODC1* to test the effect of RNA preservation method on transcript abundance. No differences were observed between RNA*later*® and liquid nitrogen preserved samples for these genes ($P > 0.20$).

RNA isolation and preparation of fluorescently labelled aRNA

Total RNA from approximately 30 mg of RNA*later*®-stabilized LD tissue from Piau foetuses was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Total RNA from three Piau foetuses per litter was pooled for use in subsequent assays. Total RNA from approximately 1.0 g of LD tissue pooled from three foetuses from each of the YL gilts was extracted using TRIzol® reagent (Invitrogen Corp.) according to the manufacturer's instructions. Total RNA concentrations were measured using a NanoDrop spectrophotometer (NanoDrop Technologies Inc.), and quality and integrity were determined with an RNA 6000 Labchip® kit using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). Total RNA (1 μg) was reverse transcribed with an oligo(dT) primer and ArrayScript™ following the Amino Allyl MessageAmp™ II aRNA Kit (Ambion Inc.) protocol. After purification, 20 μg of each allyl-modified aRNA was dye coupled using NHS ester Cy™3 and Cy™5 dyes (GE Healthcare).

Oligonucleotide microarray hybridization and image processing

Transcriptional profiling was performed using the Swine Protein-Annotated Oligonucleotide Microarray (Pigoligoarray; http://www.pigoligoarray.org). We have previously evaluated this microarray for use in pig functional genomics studies and have reported on the high quality of the array annotation (Steibel *et al.* 2009b). The array contains a total of 20 400 70-mer oligonucleotides, which includes 19 980

non-control probes representing over 18 000 pig genes. Hybridization and washing procedures were performed as previously described by our group (Steibel *et al.* 2009b). Images were processed at two different wavelengths (Cy3 and Cy5 fluorescence) to detect relative transcript abundance using an Axon GenePix® 4000B scanner (Molecular Devices) and the GENE PIX PRO 6.0 software (Molecular Devices). After spot alignment, non-normalized and non-background corrected median intensity values of each dye channel as well as normalized Cy5/Cy3 log ratios were stored as comma-separated values for analysis, and data were submitted to the National Center for Biotechnology Information's Gene Expression Omnibus database (NCBI GEO: GSE21412).

Experimental design and statistical analysis

To study the joint effects of age and breed, a factorial design was used to allow investigation of interactions between both factors. Gene expression patterns across ages and breeds were evaluated using a connected loop design (Tempelman 2005) with 13 microarray slides (Fig. S1). Dyes were balanced so that each RNA sample was measured once with each dye.

Microarray data were normalized in the LIMMA software (Dudoit & Yang 2003) using a within-array global loess normalization (Yang *et al.* 2002). The Cy3 and Cy5 log-normalized intensities were derived from the loess-normalized log Cy5/Cy3 intensities and analysed using a linear mixed model (SAS, 2007):

$$Y_{ijklm} = \mu + \text{Dye}_i + \text{Breed}_j + \text{Age}_k + \text{Breed} \times \text{Age}_{jk} + \text{Sample}(\text{Breed} \times \text{Age})_{l:jk} + \text{Array}_m + e_{ijklm},$$

where Y_{ijklm} denotes the normalized log intensity for the l^{th} sample within the j^{th} breed and k^{th} age and labelled with the i^{th} dye, μ is the overall mean, Dye_i is the fixed effect of the i^{th} dye (i = Cy3, Cy5), Breed_j is the fixed effect of the j^{th} breed (j = YL, Piau), Age_k is the fixed effect of the k^{th} age (k = 40, 70), $\text{Breed} \times \text{Age}_{jk}$ pertains to the fixed interaction effects of the j^{th} breed with the k^{th} age, $\text{Sample (Breed} \times \text{Age)}_{l:jk}$ is the random effect of the l^{th} sample (l = 1, 2, 3 for both ages of YL; l = 1, 2, 3, 4 for both ages of Piau) within the j^{th} breed and k^{th} age, Array_m is the effect of the m^{th} array (m = 1, 2 …, 13) and e_{ijklm} is the residual. The software SAS® PROC MIXED was used for this analysis (Littell *et al.* 2006). To limit the occurrence of false positives, *P*-values were adjusted using the false discovery rate (FDR) procedure outlined by Storey & Tibshirani (2003).

Genes identified to be differentially expressed (FDR ≤ 0.05) between developmental ages or between breed types were evaluated using the DAVID software (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov/; Dennis *et al.* 2003; Huang *et al.* 2009) to determine functional classifications based on gene ontology categories. Differentially expressed genes were further evaluated using the Ingenuity Pathways Analysis software (Ingenuity Systems) to identify biological pathways.

Real-time quantitative PCR (qRT-PCR) analysis

Quantitative real-time reverse transcription PCR (qRT-PCR) analyses using TaqMan® technology (Applied Biosystems) were used to confirm microarray results. Thirteen genes observed to be differentially expressed either between ages or between breeds were chosen for confirmation: *carbonic anhydrase III muscle specific* (*CA3*), *catenin (cadherin-associated protein) beta 1* (*CTNNB1*), *cathepsin L2* (*CTSL2*), *delta-like 1 homolog* (*DLK1*), *F-box protein 32* (*FBXO32*), *myozenin 1* (*MYOZ1*), *NRAP*, *ornithine decarboxylase 1* (*ODC1*), *sarcopilin* (*SLN*), *signal transducer and activator of transcription 1* (*STAT1*), *TIMP metallopeptidase inhibitor 3* (*TIMP3*), *tenascin C* (*TNC*) and *ubiquitin-specific peptidase 13 (isopeptidase T-3)* (*USP13*). Each of the thirteen assays consisted of two unlabelled PCR primers and a FAM™ dye-labelled TaqMan® MGB (minor groove binder) probe. Ten of the primer-probe sets were commercially available from Applied Biosystems, whereas the remaining three (*USP13*, *ODC1* and *TIMP3*) were custom designed by Applied Biosystems for this project (Custom TaqMan® Gene Expression Assay). *Hypoxanthine phosphoribosyltransferase 1* (*HPRT1*; Von der Hardt *et al.* 2009; Lobjois *et al.* 2008) was used as a control gene for these analyses, and transcript abundance of *HPRT1* was consistent across all samples. TaqMan® primer-probe information is provided in Table S1.

Total RNA (2 μg) from LD muscle samples (n = 14) was reverse transcribed using random primers with the High Capacity cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. Assays were performed in triplicate using 50 or 90 ng cDNA and the TaqMan 2X Universal PCR Master Mix (20 μl final volume per reaction) in an ABI Prism 7500 sequence detection system (Applied Biosystems). Cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Transcription levels for each gene were normalized using the *HPRT1* gene. The cycles to threshold (C_t) data were analysed using the %QPCR_MIXED macro developed in SAS (v. 9.1.3) based on a linear mixed model (Steibel *et al.* 2009a) and applied in this case for a completely randomized design with two factors.

Results

Identification of differentially expressed genes

Microarray data were submitted to the NCBI GEO database (GSE21412). A total of 1043 oligonucleotides with HUGO Gene Nomenclature Committee (HGNC) annotation were found to be differentially expressed (FDR ≤ 0.05) in LD

muscle of foetuses between 40 and 70 d gestation in either YL or Piau pigs. Only 268 of these oligos were differentially expressed between 40 and 70 d gestation foetuses in both breed types, whereas 261 were significant only in the YL pigs and 514 were significant only in the Piau pigs. Limiting the number of significantly differentially expressed oligos (FDR ≤ 0.05) to only those exhibiting FC differences ≥1.5 to help ensure reproducibility (Shi *et al.* 2008) resulted in 197 oligos differentially expressed between 40 and 70 d of gestation in both breed types, 85 oligos differentially expressed in YL pigs only and 204 differentially expressed in Piau pigs only (Table S2).

A total of 4112 oligos were differentially expressed (FDR ≤ 0.05) between YL and Piau breed types at either 40 or 70 d of gestation. Of these, 1728 oligos were differentially expressed between the two breed types at both 40 and 70 d of gestation, 1508 oligos were significant only at 40 d of gestation and 876 were significant only at 70 d of gestation. Limiting the number of significantly differentially expressed oligos (FDR ≤ 0.05) to only those exhibiting FC differences ≥1.5 resulted in 840 oligos differentially expressed between YL and Piau at both ages, 282 oligos differentially expressed only at 40 d of gestation and 178 differentially expressed only at 70 d of gestation (Table S2).

Gene ontology annotation

To gain insight into the biological functions of differentially expressed transcripts in LD muscle between 40 and 70 d of gestation in the YL and Piau pigs, gene ontology (GO) annotations were evaluated using the DAVID software (Dennis *et al.* 2003; Huang da *et al.* 2009). Category enrichment information based on biological process (BP), cellular component (CC) and molecular function (MF) GO terms are included in Table S3. Reduced lists are provided in Tables 1–3 for BP, CC and MF GO terms, respectively.

The GO annotation was performed by considering four sets of differentially expressed genes: genes differentially expressed between developmental ages for each breed type, and genes differentially expressed between the two breed types at each developmental age. The most highly enriched BP categories for the age comparisons in both breeds were related to the muscle system (Table 1), including muscle system process, muscle contraction, cytoskeletal organization and muscle organ development. Interestingly, the BP categories of phosphate metabolic process and phosphorus metabolic process included 22 genes for the age comparison in the YL pigs, but these categories had no differentially expressed genes for the age comparison in the Piau pigs. The most enriched BP categories for the breed comparison at 40 d of gestation were regulation of transcription and transcription. However, these categories included no differentially expressed genes either for the age comparisons within breed or for the breed comparison at 70 d of gestation.

The most enriched GO CC categories for both the age and breed comparisons were non-membrane-bound organelle and cytoskeleton (Table 2). For MF, the most enriched terms for the age comparisons were cytoskeletal protein binding and structural molecule activity (Table 3). The MF category of calcium ion binding included 31 differentially expressed genes in the age comparison for the Piau pigs; however, there were no differentially expressed genes in this category for the age comparison of the YL pigs. The most enriched MF categories for the breed comparisons included nucleotide binding and DNA binding. Thus, the GO annotation indicates that the differentially expressed genes identified in this study are categorized into functional categories that would be expected for foetal LD muscle. In addition, several GO categories were identified to be enriched with differentially expressed genes for one breed type but not for the other, suggesting breed-specific developmental gene expression patterns.

Pathway analysis

The 467 genes observed to be differentially expressed in LD muscle between 40 and 70 d of gestation in either breed type (FDR ≤ 0.05; FC ≥ 1.5) were further functionally evaluated using the Ingenuity Pathways Analysis (IPA) software. This gene set was categorized into a total of 136 networks by the IPA software. A network containing 54 genes related to skeletal and muscular system development was observed for genes that were differentially expressed between 40 and 70 d gestation in both the YL and Piau breed types (Fig. 1). In this network, 39 genes were more highly expressed at 70 d of gestation (green colour), whereas 15 genes were more highly expressed at 40 d of gestation (red colour). While the same genes were observed to exhibit increased or decreased mRNA abundance in both the Piau and YL breed types in this network, the FC differences varied for some genes, as depicted by the intensity of the green and red colours between panels (a) and (b) of Fig. 1. Interestingly, several genes exhibit higher expression at 70 d in the Piau pigs (higher green intensity), while several genes exhibit higher expression at 40 d in the YL pigs (higher red intensity), suggesting the potential for a breed type–specific expression pattern.

An additional network that included differentially expressed genes between 40 and 70 d gestation in both Piau and YL pigs was related to cellular function and maintenance (Fig. S2). For this network, 10 genes were more highly expressed at 70 d of gestation and four genes were more highly expressed at 40 d of gestation. A third informative network, cellular morphology and cellular assembly, involved 53 genes that were differentially expressed between the two ages only in the Piau breed (Fig. S3). Thirty-five of these genes exhibited increased

Table 1 Over-represented gene ontology biological processes for differentially expressed genes in longissimus dorsi muscle of Piau and Yorkshire–Landrace (YL) pigs at 40 and 70 d of gestation[1].

	Piau (40 vs. 70 d)[2]		YL (40 vs. 70 d)[3]		40 d (YL vs. P)[4]		70 d (YL vs. P)[5]	
Biological process GO term (gene ontology ID no.)	Gene count	*P*-value	Gene count	*P*-value	Gene count	*P*-value	Gene count	*P*-value
Muscle system process (3012)	30	1.17E–18	22	5.52E–14			15	8.28E–02
Muscle contraction (6936)	30	7.93E–20	22	8.20E–15			15	4.44E–02
Cytoskeleton organization (7010)	25	1.75E–05	20	2.54E–05	41	7.31E–03	39	3.82E–03
Muscle organ development (7517)	25	1.64E–11	13	7.12E–05			23	3.20E–03
Response to organic substance (10033)	24	3.13E–02	22	2.31E–03	59	2.10E–02	50	7.93E–02
Macromolecular complex subunit organization (43933)	24	2.67E–02	20	9.01E–03	69	1.62E–04	67	2.20E–05
Homeostatic process (42592)	24	4.61E–02						
Macromolecular complex assembly (65003)	23	2.43E–02	19	9.78E–03	66	1.27E–04	64	1.91E–05
Cell adhesion (7155)	23	4.01E–02						
Biological adhesion (22610)	23	4.02E–02						
Protein complex assembly (6461)	20	1.07E–02	17	3.42E–03	46	7.71E–03	43	5.59E–03
Protein complex biogenesis (70271)	20	1.07E–02	17	3.42E–03	46	7.71E–03	43	5.59E–03
Generation of precursor metabolites and energy (6091)	20	3.62E–05	16	6.47E–05	33	3.25E–03	35	1.33E–04
Cellular component morphogenesis (32989)	20	7.66E–04	14	6.19E–03	35	3.14E–02		
Phosphate metabolic process (6796)			22	5.16E–02				
Phosphorus metabolic process (6793)			22	5.16E–02				
Regulation of transcription (45449)					184	2.22E–02		
Transcription (6350)					149	3.84E–02		
Protein localization (8104)					85	3.39E–05	79	2.22E–05
Cell cycle (7049)					82	1.53E–06	71	3.16E–05
Positive regulation of macromolecule metabolic process (10604)					81	1.03E–04	72	3.80E–04
Proteolysis (6508)					79	4.79E–02	80	3.31E–03
Macromolecule catabolic process (9057)					78	2.22E–05	74	6.35E–06
Negative regulation of macromolecule metabolic process (10605)					76	8.23E–06	73	1.33E–06
Cellular macromolecule catabolic process (44265)					74	1.81E–05	71	3.31E–06
Protein transport (15031)					74	9.26E–05	70	3.29E–05
Establishment of protein localization (45184)					74	1.23E–04	70	4.34E–05

[1]Complete gene ontology lists available in Table S3.
[2]Differentially expressed genes in Piau pigs between 40 and 70 d of gestation. Categories enriched with 20 or more genes are included in the table.
[3]Differentially expressed genes in YL pigs between 40 and 70 d of gestation. Categories enriched with 20 or more genes are included in the table unless the category was included for previous contrast.
[4]Differentially expressed genes at 40 d of gestation between YL and Piau pigs. Categories enriched with 70 or more genes are included in table unless the category was included for previous contrast.
[5]Differentially expressed genes at 70 d of gestation between YL and Piau pigs. Categories enriched with 70 or more genes are included in table unless the category was included for previous contrast.

expression at 70 d of gestation, whereas 18 exhibited higher expression at 40 d of gestation.

Quantitative RT-PCR confirmation of differentially expressed genes

A total of 13 differentially expressed genes were selected for confirmation by qRT-PCR. Selected genes represented various functional groups, including myofibrillar genes (*MYOZ1* and *NRAP*), proliferation or differentiation (*DLK1* and *ODC1*), metabolic processes (*SLN* and *CA3*), extracellular matrix (*TIMP3* and *TNC*), signal transduction or transcription activation (*STAT1* and *CTNNB1*) and phosphorylation-dependent ubiquitination process or proteolysis mechanisms (*CTSL2*, *FBXO32* and *USP13*). In addition, five of these genes (*DLK1*, *NRAP*, *MYOZ1*, *SLN* and *TNC*) were included in the skeletal and muscular system development network shown in Fig. 1.

Table 2 Over-represented gene ontology cellular components for differentially expressed genes in longissimus dorsi muscle of Piau and Yorkshire–Landrace (YL) pigs at 40 and 70 d of gestation[1].

	Piau (40 vs. 70 d)[2]		YL (40 vs. 70 d)[3]		40 d (YL vs. P)[4]		70 d (YL vs. P)[5]	
Cellular component GO term (gene ontology ID no.)	Gene count	*P*-value	Gene count	*P*-value	Gene count	*P*-value	Gene count	*P*-value
Non-membrane-bounded organelle (43228)	70	4.87E−02	54	2.40E−02	222	2.27E−08	197	4.72E−07
Intracellular non-membrane-bounded organelle (43232)	70	4.87E−02	54	2.40E−02	222	2.27E−08	197	4.72E−07
Cytoskeleton (5856)	56	9.16E−06	41	1.04E−04	99	6.21E−02	91	4.73E−02
Cytoskeletal part (44430)	36	1.95E−03	29	1.03E−03	72	4.42E−02	71	7.23E−03
Cell fraction (267)	36	1.42E−02	24	8.90E−02				
Extracellular region part (44421)	35	4.07E−03						
Contractile fibre (43292)	34	2.86E−27	19	5.42E−13			15	6.59E−03
Myofibril (30016)	32	5.12E−26	18	1.51E−12			14	7.84E−03
Contractile fibre part (44449)	31	1.72E−24	19	1.60E−13			14	9.07E−03
Sarcomere (30017)	29	6.79E−24	18	1.81E−13			14	2.69E−03
Actin cytoskeleton (15629)	27	2.44E−10	19	2.61E−07	23	9.91E−02	28	1.83E−03
Vesicle (31982)	22	6.60E−02	25	1.49E−04	54	3.08E−02		
Cytoplasmic vesicle (31410)	21	7.59E−02	24	2.06E−04	50	6.25E−02		
Extracellular matrix (31012)	21	8.11E−05			42	3.17E−05	29	2.53E−02
Proteinaceous extracellular matrix (5578)	20	8.96E−05			42	5.13E−06	29	1.01E−02
Cytosol (5829)			35	3.21E−03	141	3.59E−11	128	1.90E−10
Organelle membrane (31090)			27	2.38E−02	87	8.92E−03		
Endoplasmic reticulum (5783)			22	8.04E−02	93	7.94E−06	85	1.16E−05
Cytoplasmic membrane-bounded vesicle (16023)			21	4.49E−04	48	1.21E−02	40	6.03E−02
Endomembrane system (12505)			19	6.97E−02	71	7.28E−04	55	4.78E−02
Organelle envelope (31967)			17	3.69E−02	53	1.20E−02	46	3.29E−02
Envelope (31975)			17	3.78E−02	53	1.28E−02	46	3.50E−02
Membrane-enclosed lumen (31974)					198	2.66E−16	177	2.79E−14
Organelle lumen (43233)					195	2.95E−16	174	4.06E−14
Intracellular organelle lumen (70013)					191	6.75E−16	169	2.03E−13
Nuclear lumen (31981)					156	6.46E−13	134	9.42E−10
Nucleoplasm (5654)					105	3.07E−11	88	4.92E−08
Mitochondrion (5739)					83	2.57E−02	75	3.05E−02
Golgi apparatus (5794)					71	1.08E−02	66	6.86E−03

[1]Complete gene ontology lists are available in Table S3.
[2]Differentially expressed genes in Piau pigs between 40 and 70 d of gestation. Categories enriched with 20 or more are genes included in the table.
[3]Differentially expressed genes in YL pigs between 40 and 70 d of gestation. Categories enriched with 20 or more genes are included in the table unless the category was included for previous contrast.
[4]Differentially expressed genes at 40 d of gestation between YL and Piau pigs. Categories enriched with 70 or more genes are included in the table unless the category was included for previous contrast.
[5]Differentially expressed genes at 70 d of gestation between YL and Piau pigs. Categories enriched with 70 or more genes are included in the table unless the category was included for previous contrast.

Nine of the selected genes exhibited differential expression in LD between 40 and 70 d of gestation in at least one breed type using the Pigoligoarray, and expression patterns for all of these genes were confirmed by qRT-PCR ($P \leq 0.01$; Fig. 2). *CA3*, *DLK1*, *MYOZ1*, *NRAP* and *SLN* all exhibited higher mRNA abundance at 70 d of gestation in both the YL and Piau breed types on the microarray, and these expression patterns were confirmed by qRT-PCR. Expression of *TNC* was observed to be higher in LD at 40 d of gestation in both YL and Piau pigs on the microarray, and this was also confirmed by qRT-PCR. Microarray results for *CTSL2* indicated higher expression at 40 d of gestation for Piau pigs, but no difference between ages for YL pigs, and this result was confirmed with the qRT-PCR assay. *FBXO32* and *USP13* exhibited higher expression at 70 d of gestation in Piau pigs on the microarray, but no significant difference was observed for the YL pigs using FDR ≤ 0.05. Results of the qRT-PCR analyses for these genes confirmed that expression was higher in LD at 70 d for the Piau pigs and also indicated expression to be higher at 70 d for the YL pigs.

Results for qRT-PCR analyses of six genes that were shown by microarray analysis to be significantly differentially expressed in LD at either 40 or 70 d of gestation or at both ages when comparing YL and Piau pigs are shown in Fig. 3. This gene set includes two of the genes that were also

Table 3 Over-represented gene ontology molecular functions for differentially expressed genes in longissimus dorsi muscle of Piau and Yorkshire–Landrace (YL) pigs at 40 and 70 d of gestation[1].

	Piau (40 vs. 70 d)[2]		YL (40 vs. 70 d)[3]		40 d (YL vs. P)[4]		70 d (YL vs. P)[5]	
Molecular function GO term (gene ontology ID no.)	Gene count	*P*-value	Gene count	*P*-value	Gene count	*P*-value	Gene count	*P*-value
Cytoskeletal protein binding (8092)	40	7.05E–13	24	2.61E–06	57	3.96E–06	53	2.60E–06
Structural molecule activity (5198)	35	2.80E–07	23	2.69E–04				
Calcium ion binding (5509)	31	7.96E–03						
Actin binding (3779)	28	7.20E–10	18	1.04E–05	44	6.64E–07	39	3.70E–06
Identical protein binding (42802)	23	1.28E–02	22	7.72E–04				
Nucleotide binding (166)			48	1.23E–02	184	1.69E–06	168	9.36E–07
Purine nucleotide binding (17076)			42	1.43E–02	138	1.01E–02	126	6.18E–03
Ribonucleotide binding (32553)			42	6.97E–03	134	7.31E–03	122	4.97E–03
Purine ribonucleotide binding (32555)			42	6.97E–03	134	7.31E–03	122	4.97E–03
Nucleoside binding (1882)			35	3.04E–02			100	6.07E–02
Purine nucleoside binding (1883)			34	4.33E–02			99	6.22E–02
Adenyl ribonucleotide binding (32559)			33	3.01E–02	102	9.09E–02	92	8.28E–02
ATP binding (5524)			33	2.55E–02	101	8.75E–02		
Adenyl nucleotide binding (30554)			33	5.53E–02			96	9.06E–02
DNA binding (3677)					165	8.66E–03	138	8.90E–02
Transcription regulator activity (30528)					116	2.76E–03	98	2.36E–02
RNA binding (3723)					85	1.14E–09	71	3.97E–07

[1]Complete gene ontology lists are available in Table S3.

[2]Differentially expressed genes in Piau pigs between 40 and 70 d of gestation. Categories enriched with 20 or more genes are included in the table.

[3]Differentially expressed genes in YL pigs between 40 and 70 d of gestation. Categories enriched with 20 or more genes are included in the table unless the category was included for previous contrast.

[4]Differentially expressed genes at 40 d of gestation between YL and Piau pigs. Categories enriched with 70 or more genes are included in the table unless the category was included for previous contrast.

[5]Differentially expressed genes at 70 d of gestation between YL and Piau pigs. Categories enriched with 70 or more genes are included in the table unless the category was included for previous contrast.

differentially expressed between ages (*FBXO32* and *NRAP*). Differential expression of most of these genes was confirmed. Expression of *NRAP* was observed to be higher in LD of Piau pigs at both 40 and 70 d of gestation on the microarray, and this result was confirmed by qRT-PCR. *FBXO32* exhibited significantly higher expression in LD from Piau pigs than in LD from YL pigs at 70 d of gestation, but no difference in expression between breed types was observed at 40 d of gestation, and this result was also confirmed by qRT-PCR. Results for *ODC1* on the microarray indicated that expression was higher in Piau pigs at 70 d of gestation, but no breed-type differences were observed at 40 d of gestation with an FDR ≤ 0.05. Results for *ODC1* using qRT-PCR confirmed higher expression in Piau pigs at 70 d of gestation and also indicated that expression was significantly higher in Piau pigs at 40 d of gestation. Similarly, microarray results for *TIMP3* indicated that *TIMP3* expression was higher in Piau pigs at 40 d of gestation, but breed-type differences were not significant at 70 d of gestation (FDR ≤ 0.05). Results for qRT-PCR analysis of *TIMP3* revealed significantly higher expression of *TIMP3* in LD of Piau pigs at both 40 and 70 d of gestation. On the microarray, *CTNNB1* exhibited a 4.1-fold higher expression in YL pigs than Piau pigs at 40 d of gestation, with no breed difference observed at 70 d of gestation. There was also no difference in expression as observed by qRT-PCR analysis between breed types for this gene at 70 d. However, qRT-PCR failed to confirm the breed difference that were seen with the microarray in the 40 d samples. *STAT1* exhibited significantly higher expression in Piau pigs at both 40 and 70 d of gestation by qRT-PCR analysis. However, this result was opposite to the *STAT1* expression pattern observed using the Pigoligoarray. On both platforms, transcript abundance levels for *CTNNB1* and *STAT1* were relatively low, which could account for the inconsistent results for these genes.

Discussion

This study evaluated transcriptional profiles in LD muscle tissue of pigs at 40 and 70 d of gestation, which are two developmental stages that encompass the primary and secondary waves of muscle fibre formation in pigs (Wigmore & Stickland 1983; Wigmore & Evans 2002). Samples obtained from YL cross-bred pigs and Piau pigs (a native Brazilian breed), which are breed types that differ in muscularity, were used to allow breed-type comparisons. Our results revealed a large number of differentially expressed

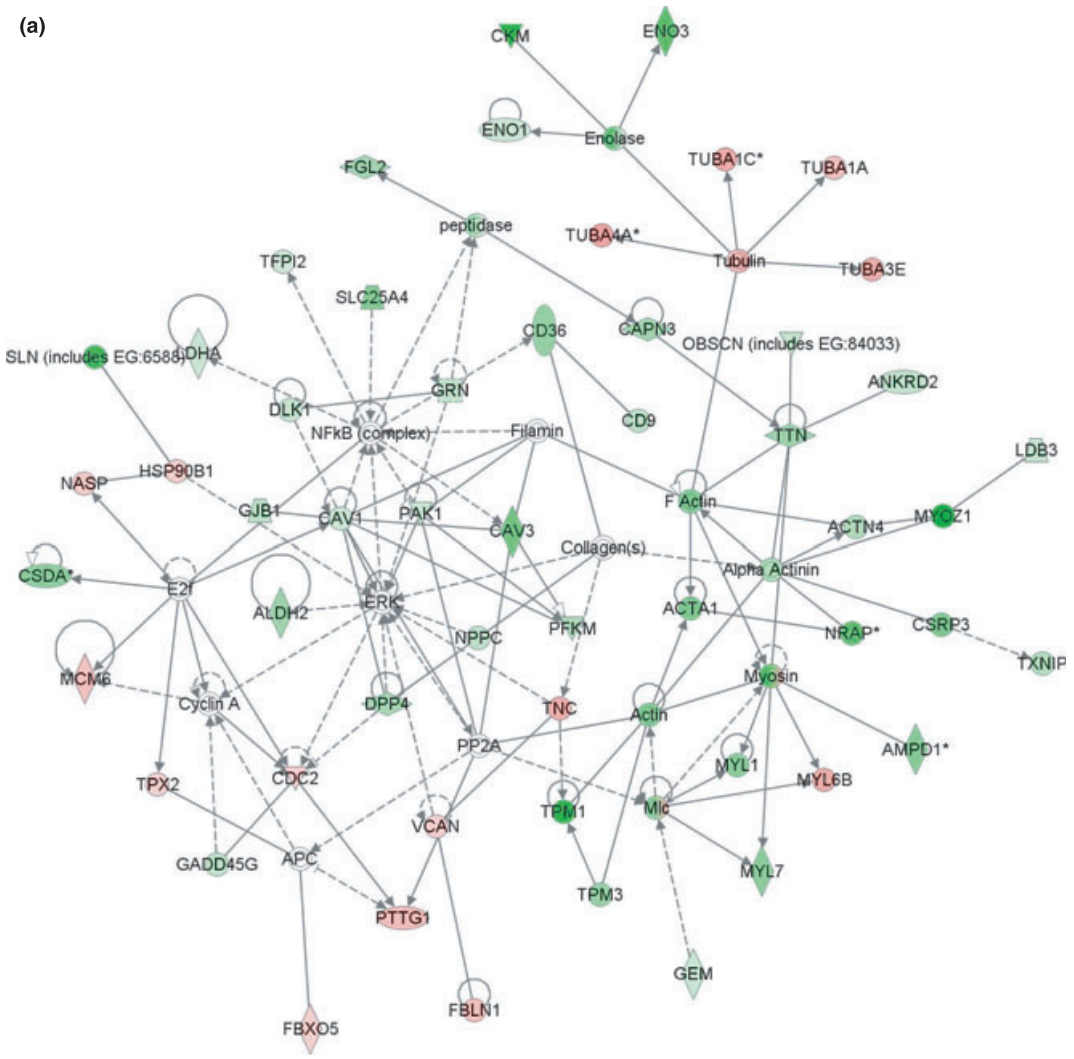

Figure 1 Skeletal and muscular system development and function gene network containing 54 genes differentially expressed in longissimus dorsi (LD) muscle between 40 and 70 d of gestation derived using the Ingenuity Pathways Analysis System software (Ingenuity Systems®). (a) Differentially expressed genes in Piau pigs. (b) Differentially expressed genes in Yorkshire–Landrace (YL) cross-bred pigs. Red, higher mRNA abundance in LD muscle at 40 d of gestation relative to 70 d of gestation; Green, higher mRNA abundance in LD muscle at 70 d of gestation relative to 40 d of gestation. Darker colours signify higher fold change expression differences.

genes both between developmental ages and between breed types. In general, the developmental transcript profiles for the Piau and YL pigs were similar, although breed-specific patterns of gene expression were revealed. In addition, the relative abundance of transcripts (based on fluorescence intensity using the microarray) tended to be greater for the Piau pigs at 70 d of gestation, suggesting that gene expression in LD muscle of YL pigs may be more delayed than in Piau pigs. This observation is consistent with the results of Cagnazzo *et al.* (2006), who reported a study examining gene expression in developing skeletal muscle of Duroc and Pietrain pigs, which are breeds that differ for muscle fibre characteristics as well as growth and muscularity phenotypes. These researchers observed differential expression for numerous myogenesis-related genes and suggested that pigs of the heavier muscled Pietrain breed may exhibit a delayed myogenesis process, perhaps resulting in a larger pool of myogenic satellite cells available for muscle hypertrophy, or possibly increased numbers of myofibres, relative to pigs of the Duroc breed.

Following global transcript profiling analysis using the microarray, several genes were selected for confirmation by qRT-PCR. Some of these genes encode products with functions related to skeletal muscle structure or function. Myozenin proteins including MYOZ1 appear to serve as intracellular binding proteins involved in linking Z-disc proteins such as *alpha*-actinin and titin-cap, and MYOZ1 plays a complex role in the modulation of calcineurin signalling (Faulkner *et al.* 2000). We observed significantly higher expression of *MYOZ1* at 70 d of gestation relative

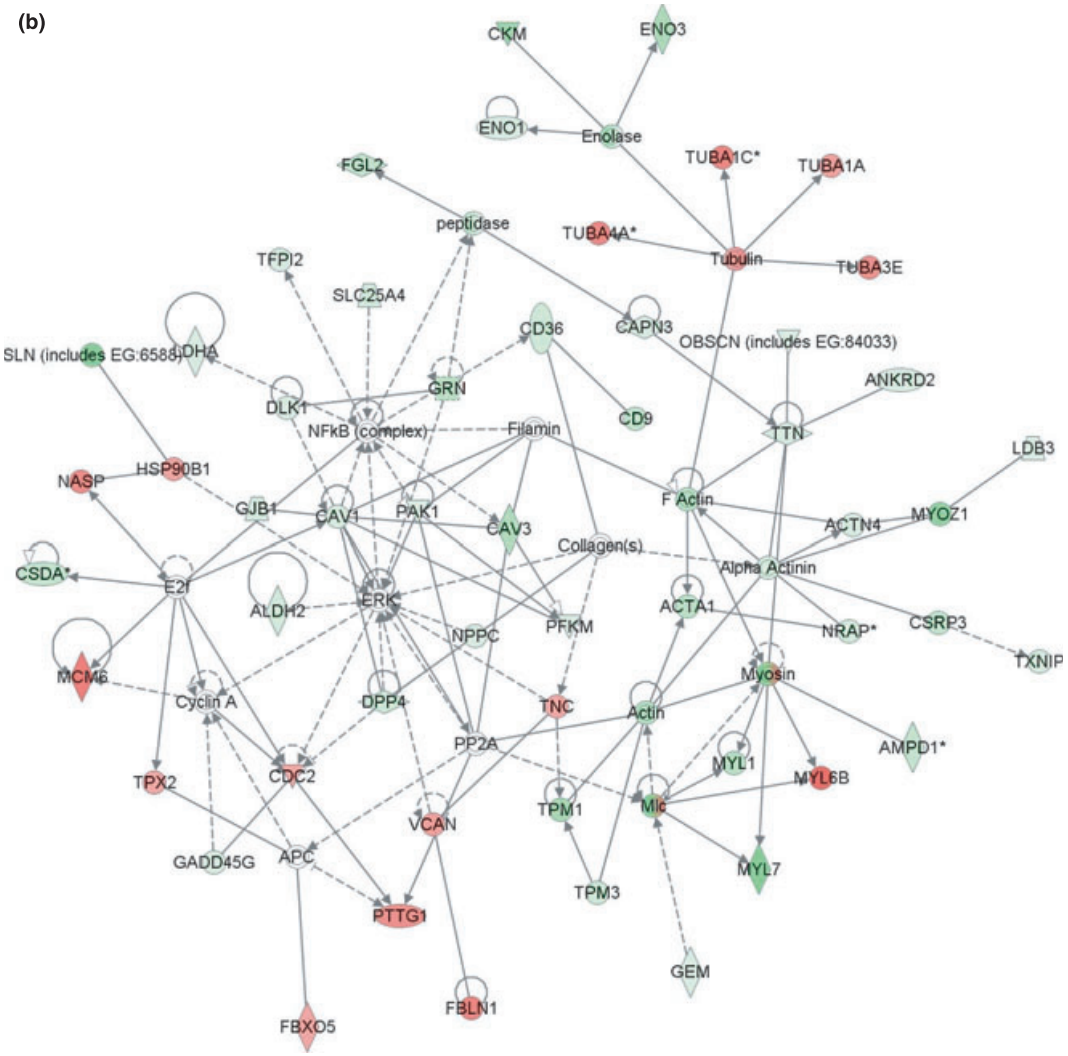

Figure 1 (Continued)

to 40 d of gestation in both Piau and YL pigs. This is consistent with recent observations by Raymond *et al.* (2010), who compared gene expression patterns in human skeletal muscle tissue and cultured myotubes, and found *MYOZ1* levels to be significantly downregulated in cultured myotubes, supporting the role of this gene in tissue structure and maturation. The product of the *NRAP* gene is associated with developing skeletal muscle myofibrillar structures (Lu *et al.* 2008). Our results indicated that *NRAP* expression was higher at 70 d of gestation in both Piau and YL pigs and also that expression was higher in Piau pigs at both ages. Similar results were reported by Muráni *et al.* (2007), who observed higher expression of *NRAP* in skeletal muscle of Pietrain foetuses at 35 d of gestation when compared to Duroc foetuses. The *SLN* gene encodes a small proteolipid that regulates several sarcoplasmic reticulum Ca(2 +)-ATPases. Our results indicated that *SLN* expression was higher at 70 d of gestation in both Piau and YL pigs, with highest expression in the Piau pigs. This agrees in results reported by Lin & Hsu (2005), who compared neonatal pigs of a native breed (Taoyuan) to Duroc pigs and observed higher *SLN* expression in the Taoyuan pigs.

Additional genes selected for qRT-PCR confirmation encode products involved in cellular or tissue growth. The tissue inhibitors of metalloproteinsases (TIMP) were originally characterized as inhibitors of matrix metalloproteinases, but it is now known that they have a much wider range of biological activities, including effects on cell growth and differentiation, cell migration and apoptosis, among others (Brew & Nagase 2010). We observed higher expression of *TIMP3* in Piau pigs vs. YL pigs at both 40 and 70 d of gestation, indicating a breed type–specific expression pattern for this gene. Similarly, we also observed higher expression of *ODC1* in Piau pigs at both 40 and 70 d of gestation. The *ODC1* gene encodes the

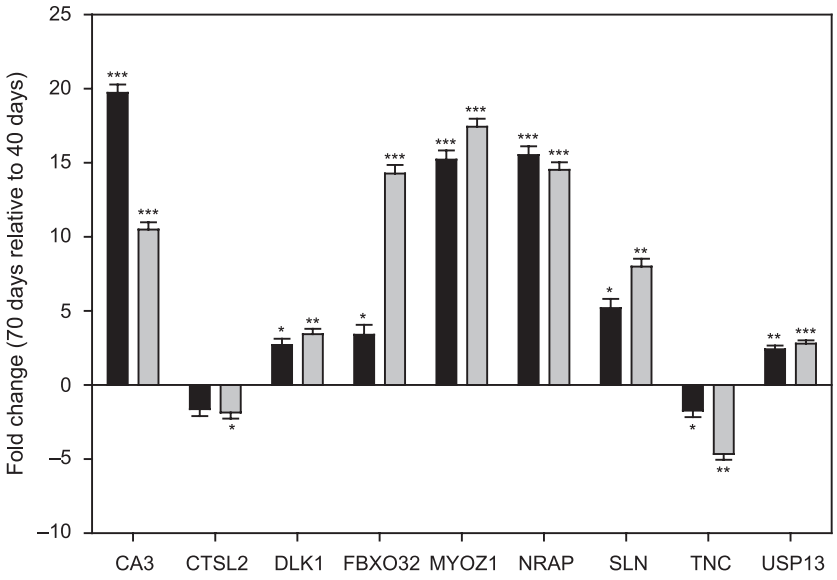

Figure 2 Quantitative RT-PCR results for nine genes observed by microarray analysis to exhibit differential expression in longissimus dorsi muscle between 40 and 70 d of gestation. Results are presented as fold changes for expression at 70 d of gestation relative to expression at 40 d of gestation, such that bars above the origin indicate higher expression at 70 d and bars below the origin indicate lower expression at 70 d. Black bars, Yorkshire–Landrace (YL) pigs; Grey bars, Piau pigs. $*P \leq 0.01$; $**P \leq 0.001$; $***P \leq 0.0001$.

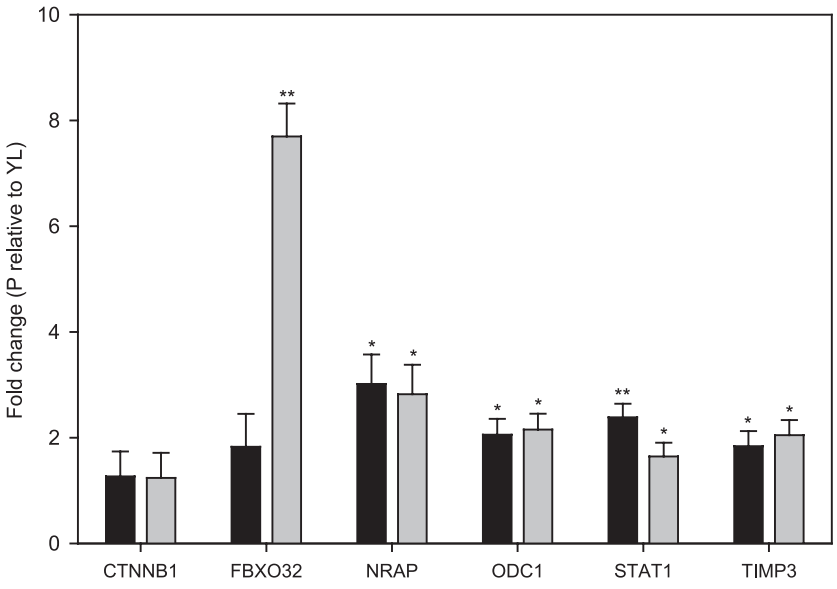

Figure 3 Quantitative RT-PCR results for six genes observed by microarray analysis to exhibit differential expression in foetal longissimus dorsi muscle between Yorkshire–Landrace (YL) and Piau pigs. Results are presented as fold changes for expression in Piau pigs relative to expression in YL pigs, such that bars above the origin indicate higher expression in Piau pigs. Black bars, 40 d of gestation; Grey bars, 70 d of gestation. $*P \leq 0.01$; $**P \leq 0.001$.

rate-limiting enzyme in the polyamine biosynthesis pathway. MacLean *et al.* (2008) found *ODC1* expression to be decreased in skeletal muscle of androgen receptor knockout mice, and based on their other microarray results and observations of muscle mass, they concluded that androgens promote muscle growth by maintaining myoblasts in the proliferative state and by delaying differentiation. While it is not possible to determine whether hormonal influences are involved in the breed-specific gene expression patterns that we observed, these observations taken together with the potential delay in gene expression for the YL pigs in this study and the Pietrain pigs in the Cagnazzo *et al.* (2006) study may support the possibility of a prolonged proliferative period, which could result in increased numbers of primary and secondary fibres or alternatively in a larger pool of myogenic satellite cells, as suggested by Cagnazzo *et al.* (2006).

The *CA3* gene is the muscle-specific member of a multigene family encoding metalloenzymes that catalyse the reversible hydration of carbon dioxide. Our results indicate that *CA3* expression was higher at 70 d of gestation than at 40 d of gestation in both breed types, which is in agreement with the results of Wang *et al.* (2006), who reported expression of *CA3* to increase in skeletal muscle from 33 to 65 d of gestation in Tongcheng pigs. The *DLK1* gene was observed to exhibit higher expression at 70 d of gestation in both breed types. This gene, located in an imprinted region of mammalian genomes, is involved in differentiation, and it

has been shown to be upregulated in skeletal muscle of callipyge lambs that exhibit extreme muscle hypertrophy (Fleming-Waddell *et al.* 2007). The *TNC* gene product is a mechano-regulated, morphogenic, extracellular matrix protein that is associated with tissue remodelling (Flück *et al.* 2008), and it is involved in innervation during development. We observed *TNC* to be more highly expressed at 40 d of gestation in both Piau and YL pigs, suggesting a greater role for *TNC* during primary fibre formation.

Two genes selected for qRT-PCR analysis did not confirm the results observed with the microarray. The *CTNNB1* gene product is a primary mediator of the WNT/β-catenin signalling pathway that when activated leads to the stabilization of β-catenin, which enters the nucleus to activate target genes including *MYOD* and *MYF5* (Armstrong & Esser 2005), thus potentially increasing myoblast proliferation. Expression of *CTNNB1* was significantly higher in YL pigs vs. Piau pigs on the microarray at 40 d of gestation, although no breed difference was observed at 70 d of gestation. Using qRT-PCR, no significant breed-type difference was observed for this gene at either age. We detected relatively low signal intensities for this gene on both platforms, which could account for the inconsistent results. Cagnazzo *et al.* (2006) observed *CTNNB1* to have increased expression in Pietrain foetuses at several developmental ages from 35 to 91 d of gestation compared to Duroc foetuses. Thus, this gene may exhibit breed-specific expression patterns, and further study may be warranted. The *STAT1* gene encodes a protein that is phosphorylated and acts as a transcriptional activator through a signal transduction pathway mediated by interferons. Sun *et al.* (2007) reported that *STAT1* is an essential part of a *JAK1-STAT1-STAT3* signalling pathway that promotes myoblast proliferation, supporting a role for this gene in foetal muscle development. On the microarray, *STAT1* exhibited significantly higher expression in LD muscle of YL pigs. However, results of the qRT-PCR assay indicated that expression of this gene was significantly higher in the Piau pigs. Low signal intensities on both platforms may have contributed to the inconsistent results.

Three genes selected for qRT-PCR confirmation are known to function in proteolysis pathways (*CTSL2*, *FBXO32* and *USP13*). Ubiquitin-specific proteases are enzymes that remove ubiquitin from specific protein substrates and allow protein salvage from proteosome degradation or regulation of protein localization or activation. Little information is available regarding the specific function of *USP13*; however, the mRNA abundance of this gene is higher in skeletal muscle than in any other tissue in mice and humans (http://biogps.gnf.org). We observed higher expression of *USP13* in LD muscle at 70 d of gestation in both Piau and YL pigs, suggesting an involvement of this protein in the formation of secondary muscle fibres. *CTSL2* encodes a lysosomal cysteine proteinase, and this gene has been shown to be induced as a result of muscle atrophy (Lecker *et al.* 2004). We observed increased expression of *CTSL2* at 40 d of gestation in Piau pigs, but found no differences in expression between ages for YL pigs. Thus, while additional studies are needed, this gene may be involved in protein turnover, and developmental regulation of gene expression may be breed specific. *FBXO32* (also known as *atrogin-1 and muscle atrophy F-box, MAFbx*) encodes an F-box-containing ubiquitin protein ligase that was identified in mice and rats because of its high level of expression during muscle atrophy (Bodine *et al.* 2001; Gomes *et al.* 2001). Tintignac *et al.* (2005) also reported a role for *FBXO32* in muscle differentiation by determining that FBXO32 mediates degradation of MYOD via the ubiquitin proteosome pathway. We observed higher expression of *FBXO32* in LD at 70 d of gestation in both Piau and YL pigs and also found that Piau pigs exhibited significantly higher expression of *FBXO32* than YL pigs at 70 d of gestation, suggesting that expression of this gene is developmentally regulated and breed type specific. These results support a role for the ubiquitin proteosome system in foetal skeletal muscle development.

In summary, we have used a whole-genome microarray (Pigoligoarray) to examine transcriptional profiles in LD muscle at 40 and 70 d of gestation in Piau and YL cross-bred pigs. These gestational ages encompass the two waves of primary and secondary muscle fibre formation in pigs. We have identified both developmental and breed type–specific patterns of gene expression. In addition, this study is the first report to evaluate gene expression in Brazilian native Piau pigs. Skeletal muscle development involves the synchronized expression and interaction of many genes, and this study provides additional insight into the process of myogenesis in pigs.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1 Loop design used for the microarray experiment.

Figure S2 Cellular Function and Maintenance gene network containing 14 genes differentially expressed in LD muscle between 40 and 70 days of gestation in Piau and Yorkshire–Landrace (YL) cross-bred pigs derived using the Ingenuity Pathways Analysis System software (Ingenuity Systems®).

Figure S3 Cellular Morphology and Cellular Assembly gene network containing 53 genes differentially expressed in LD muscle between 40 and 70 days of gestation in Piau pigs derived using the Ingenuity Pathways Analysis System software (Ingenuity Systems®).

Table S1 Primer information for genes selected for qRT-PCR.

Table S2 Lists of differentially expressed genes in longissimus dorsi muscle of Piau or Yorkshire–Landrace pigs at 40 or 70 days of gestation.

Table S3 Lists of gene ontology categories for differentially expressed genes in longissimus dorsi muscle of Piau or Yorkshire–Landrace pigs at 40 or 70 days of gestation.

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