Analysis of mRNA expression of *CNN3*, *DCN*, *FBN2*, *POSTN*, *SPARC* and *YWHAQ* genes in porcine foetal and adult skeletal muscles

K. Bílek[1], A. Knoll[1,2], A. Stratil[2], K. Svobodová[1], P. Horák[2], R. Bechyňová[2], M. Van Poucke[3], L.J. Peelman[3]

[1]Department of Animal Morphology, Physiology and Genetics, Mendel University of Agriculture and Forestry, Brno, Czech Republic
[2]Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Liběchov, Czech Republic
[3]Department of Animal Nutrition, Genetics, Breeding and Ethology, Ghent University, Merelbeke, Belgium

ABSTRACT: Skeletal muscle growth is determined by the number of prenatally formed fibres and by the degree of their postnatal hypertrophy; i.e. prenatal development may influence the postnatal growth. Suppression subtractive hybridization (SSH) was used to identify genes more expressed in fetal hind limb muscles of Piétrain pigs (44 days of gestation) compared to the adult biceps femoris. Six potential functional candidate genes (*CNN3*, *DCN*, *FBN2*, *POSTN*, *SPARC* and *YWHAQ*) were selected to verify the SSH results using real-time RT-PCR. Expression levels of the studied genes were significantly higher ($P < 0.05$) in the fetal muscle compared to the adult muscle. *FBN2* and *POSTN* exhibited the highest mRNA levels (mean relative ratios were 182.7 and 121.6, respectively). The studied genes may play an important role in muscle biology and may be candidates for muscling traits.

Keywords: mRNA; fetus; gene expression; real time RT-PCR; skeletal muscle

The potential for muscle growth in mammals largely depends on the number of the prenatally formed fibres and on the degree of their postnatal hypertrophy (Rehfeldt et al., 2000). Muscle fibre development is suggested to be regulated mainly genetically, and variation in genes controlling the muscle development and their expression can be the basis for genetic improvement of meat production.

In numerous studies, hundreds of genes expressed in porcine muscle tissues were identified and their expression levels during embryonic and fetal development were investigated (e.g. Davoli et al., 2002; te Pas et al., 2005; Cagnazzo et al., 2006). Many of the genes were mapped (Davoli et al., 2002) and some of them were used as functional or positional candidates for association studies with meat quality and carcass traits (Wimmers et al., 2007) or for QTL mapping (Geldermann et al., 2003).

To understand the biology of myogenesis, the studies of expression levels of genes in various periods of muscle development are needed. The commonly used methods for the quantification of transcription are northern blotting, microarray, and reverse transcription polymerase chain reaction (RT-PCR). Although northern blotting and

Supported by the Czech Science Foundation (Grants Nos. 523/03/H076 and 523/06/1302) and IRP IAPG (No. AV0Z50450515).

microarray are routinely used for quantification of transcription, the most sensitive methods are RT-PCR and real-time RT-PCR. A critical step in gene expression analysis is the validation of RT-PCR data normalization. The normalization is usually achieved via comparing expression profiles of the genes of interest with constitutively expressed genes known as reference or housekeeping genes. Expression stability of a greater number of porcine reference genes using RT-PCR was studied by Erkens et al. (2006). It was shown that more than one reference gene should be used for the accurate normalization of expression data (Vandesompele et al, 2002; Erkens et al., 2006).

We used suppression subtractive hybridization (Diatchenko et al., 1996) for identifying genes that are more expressed in porcine fetal than in adult muscles (Stratil et al., 2008). To verify the results of subtractive hybridisation, we studied mRNA expression levels for six selected genes in fetal and adult muscles: cytoskeletal structural protein *CNN3* (calponin 3, acidic), structural protein *DCN* (decorin; bone proteoglycan II), constitutive element of extracellular microfibrils *FBN2* (fibrillin 2), adhesion extracellular matrix protein *POSTN* (periostin; osteoblast specific factor 2), regulation protein *SPARC* (secreted protein, acidic, cysteine rich; osteonectin) and regulation protein *YWHAQ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta isoform).

MATERIAL AND METHODS

The analysis of mRNA expression was performed in the muscle samples of three Piétrain sows and their five fetuses. Samples from the hind limb muscles of the foetuses (44 days of gestation) and from the *m. biceps femoris* of adult sows were collected and stored in RNAlater (QIAGEN, Hilden, Germany) at –20°C. Homogenization of the samples was carried out in FastPrep FP 120 (ThermoSavant, Holbrook, NY, USA) and total RNA was isolated using FastRNA Pro Green Kit (Q-BIOgene, Solon, OH, USA). cDNA was synthesized from the total RNA with Omniscript RT Kit (QIAGEN, Hilden,

Table 1. Information on primers used for real-time PCR

Gene	Primer sequence (5´-3´)	Amplicon size (bp)	GenBank access No. or reference
FBN2	ATGAACCCAGACCCACTGC GAACCAAGGCCAGAAAGATTG	97	AM503091
YWHAQ	ATCCAGAACTTGCCTGCACA AAGCAACTGCATGATGAGGGT	113	AM503090
CNN3	AGATGGGCACCAACAAAGG CGAGTTGTCCACGGGTTGT	104	AM490167
DCN	AATGGATTGAACCAGATGATCG TGCGGATGTAGGAGAGCTTCT	109	NM_213920
POSTN	ATTCCTGATTCTGCCAAACAAG AGAAAATGCGTTATTCACAGGC	147	AY880669
SPARC	CAAGAACGTCCTGGTCACCTT CGCTTCTCATTCTCGTGGATC	102	AM490166
ACTB[1]	CATCAGGAAGGACCTCTACGC GCGATGATCTTGATCTTCATGG	129	DQ452569
TOP2B[1]	CTAATGATGCTGGTGGCAAAC CCGATCACTCCTAGCCCAG	100	AF222921
HPRT1[1]	AAGGACCCCTCGAAGTGTTG CACAAACATGATTCAAGTCCCTG	122	NM_001032376
PPIA[1]	GCACTGGTGGCAAGTCCAT AGGACCCGTATGCTTCAGGA	71	AY008846 (Vallee et al., 2003)

[1]reference genes

Germany) and Oligo(dT)$_{20}$ Primers (Invitrogen, Carlsbad, CA, USA). The PCR primers were designed (Table 1) using the Primer Express software v2 (Applied Biosystems, Foster City, CA, USA), except for *PPIA* (Vallee et al., 2003). Real-time PCR was performed in the 7500 Real-Time PCR System using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The real-time PCR program started with 2 min AmpErase Uracil N-glycosylase (Applied Biosystems, Foster City, CA, USA) incubation step at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. PCR efficiency of each primer pair was verified by constructing the relative standard curve. Amplification was performed in duplicate and a blank was incorporated for each gene. For the normalization of gene expression levels, geNorm application was used (Vandesompele et al., 2002). The raw data from real-time PCR were analyzed with the qBase application (Hellemans et al., 2007).

RESULTS AND DISCUSSION

For the normalization of gene expression levels, four candidate reference genes were compared: *ACTB* (actin, beta) – cytoskeletal structural protein, *HPRT1* (hypoxanthine guanine phosphoribosyltransferase 1) – purine synthesis in salvage pathway, *PPIA* (peptidyl-prolyl isomerase A) – transport protein, and *TOP2B* (topoisomerase II beta) – regulation enzyme. Special attention was paid to the selection of genes that belong to different functional classes, which significantly reduces the probability that the genes might be co-regulated. The average cycle threshold values (the fractional PCR cycle at which the fluorescent signal significantly rises above the background signal) of duplicate reactions were converted to relative quantities and these were analysed using the geNorm algorithm, which is based on the principle that the expression ratio of two ideal reference genes should be identical in all samples (Vandesompele et al., 2002). Using the geNorm algorithm, two candidate reference genes, *HPRT1* and *PPIA*, were chosen as the most stable reference genes for a subsequent analysis (Figure 1). To determine the number of reference genes required for accurate normalization the pairwise variation analysis was used between the normalization factors NFn and NFn+1. The use of another control gene than *HPRT1* and *PPIA* resulted in an increase in the inaccuracy of normalisation.

Using the real-time RT-PCR, we identified differences in the expression of the six selected genes (*CNN3*, *DCN*, *FBN2*, *POSTN*, *SPARC* and *YWHAQ*) in the porcine fetal and adult muscles. Figure 2 shows expression differences in muscle mRNA for these genes between the rescaled, normalized data of three sows and their foetuses. The 95% confidence intervals (represented by error bars) indicated that the mRNA expression le-vels of the investigated genes were significantly higher in fetuses ($P < 0.05$) than in the adult muscle tissue. The mean relative ratios were approximately

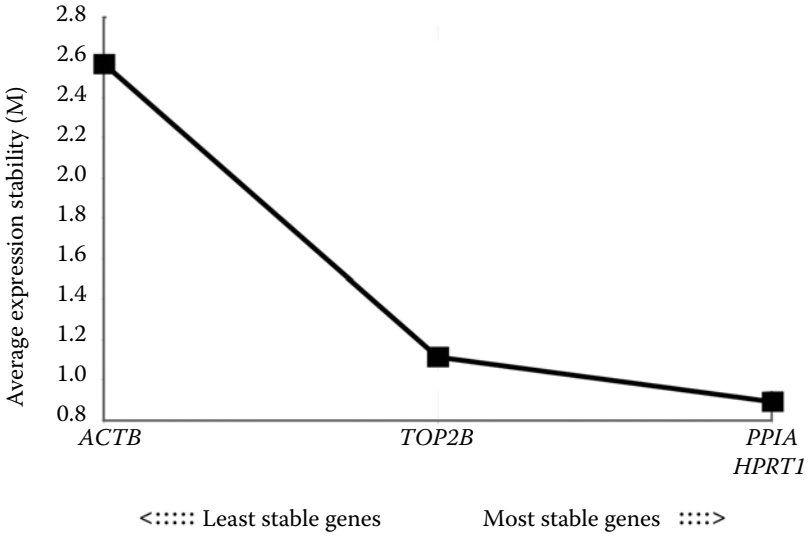

Figure 1. Average mRNA expression stability values (M) of reference genes according to geNorm

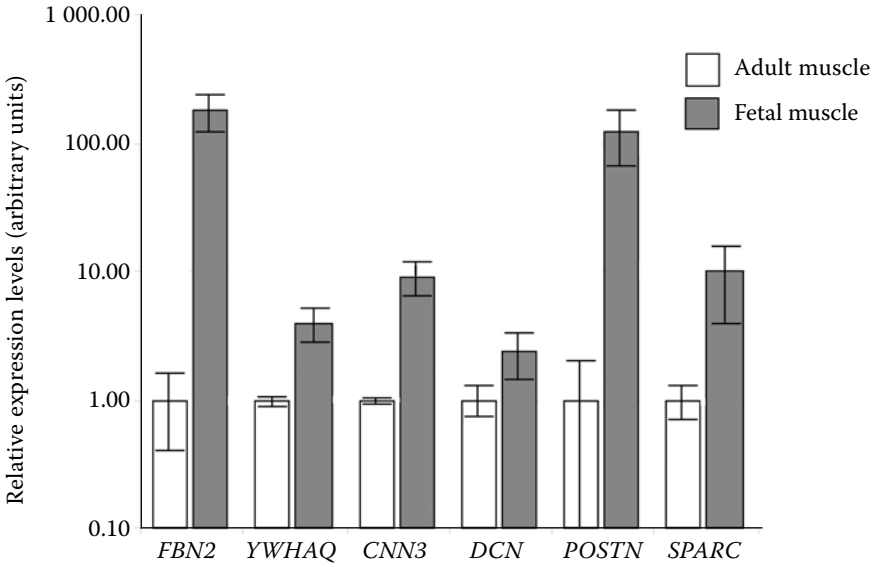

Figure 2. Relative normalized and rescaled expression of the analysed genes in adult and foetal muscle tissue, error bars represent the 95% confidence interval

8.9, 2.4, 182.7, 121.6, 9.9 and 4.0 for *CNN3*, *DCN*, *FBN2*, *POSTN*, *SPARC* and *YWHAQ*, respectively.

Expression analyses of muscle genes in different pig breeds and different developmental stages showed to be very useful both to improve gene maps and to increase the number of candidate genes that could be involved in controlling production traits (Wimmers et al., 2004). The macro- and microarray technologies were used to simultaneously measure the expression of a large number of genes in different developmental stages of embryonic/fetal development and in postnatal muscle, as well as to study differences in expression between different breeds of pigs (te Pas et al., 2005; Cagnazzo et al., 2006; Wimmers et al., 2007). It was shown that some genes are down regulated, others up regulated, and some do not change their expression level. A more sensitive method to study the expression of genes is real-time RT-PCR, which, however, is more laborious and cannot be applied to a great number of genes simultaneously. On the other hand, the limits of detection are much lower and the results are more accurate providing proper reference genes are used (Erkens et al., 2006).

The genes we studied were more expressed in the fetal muscle. Therefore, they can play important roles in muscle development and could be considered candidate genes for muscle growth. Moreover, this assumption is supported by previous reports on the functions of the genes. The *CNN3* gene participates in the formation, arrangement and disassembly of cytoskeletal structures containing both actin and myosin (Maguchi et al., 1995). *DCN* is able to regulate growth factor activity, collagen fibrogenesis, receptor tyrosine kinase activity, cancer growth etc. (Goldoni et al., 2004). Zhang et al. (1994) presented the hypothesis that *FBN2* may influence the formation and maintenance of extracellular microfibrils. The *POSTN* gene is preferentially expressed in the periosteum and peridontal ligament. It plays a role in bone and tooth formation, maintenance of their structure and it is involved in cell adhesion (Horiuchi et al., 1999). Goldblum et al. (1994) proposed that *SPARC* regulates the endothelial barrier function through F-actin-dependent changes in the cell shape, coincident with the appearance of intercellular gaps that provide a paracellular pathway for the extravasation of macromolecules. The *YWHAQ* gene is involved in the regulation of the cell-cycle via degradation of $p27^{Kip1}$, one of the CDKs activity suppressors (Fujita et al., 2002).

The genes were mapped (Stratil et al., 2008) and some QTL were found in the region of *CNN3*, *DCN* and *SPARC* (Čepica et al., 2003; Lee et al., 2003; Pierzchala et al., 2003).

It is obvious from previous studies that mammalian myogenesis and muscle growth are very complex processes. Further analyses are needed in order to better understand the biology of muscle and to identify genes and mutations involved in meat production and its variation.

REFERENCES

Cagnazzo M., te Pas M.F.W., Priem J., de Wit A.A.C., Pool M.H., Davoli R., Russo V. (2006): Comparison of prenatal muscle tissue expression profiles of two pig breeds differing in muscle characteristics. J. Anim. Sci., 84, 1–10.

Čepica S., Stratil A., Kopečný M., Blažková P., Schröffel J. jr., Davoli R., Fontanesi L., Reiner G., Bartenschlager H., Moser G., Geldermann H. (2003): Linkage and QTL mapping for *Sus scrofa* chromosome 4. J. Anim. Breed. Genet., 120, 28–37.

Davoli R., Fontanesi L., Zambonelli P., Bigi D., Gellin J., Yerle M., Milc J., Braglia S., Cenci V., Cagnazzo M., Russo V. (2002): Isolation of porcine expressed sequence tags for the construction of a first genomic transcript map of the skeletal muscle in pig. Anim. Genet., 33, 3–18.

Diatchenko L., Lau Y.-F.C., Campbell A.P., Chenchik A., Moqadam F., Huang B., Lukyanov S., Lukyanov K., Gurskaya N., Sverdlov E.D., Siebert P.D. (1996): Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc. Nat. Acad. Sci. USA, 93, 6025–6030.

Erkens T., Van Poucke M., Vandesompele J., Goossens K., Van Zeveren A., Peelman L.J. (2006): Development of a new set of reference genes for normalization of real-time RT-PCR data of porcine backfat and *longissimus dorsi* muscle, and evaluation with *PPARGCIA*. BMC Biotechnol., 6, 41 pp.

Fujita N., Sato S., Katayama K., Tsuruo T. (2002): Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization. J. Biol. Chem., 277, 28706–28713.

Geldermann H., Müller E., Moser G., Reiner G., Bartenschlager H., Cepica S., Stratil A., Kuryl J., Moran C., Davoli R., Brunsch C. (2003): Genome-wide linkage and QTL mapping in porcine F2 families generated from Pietrain, Meishan and Wild Boar crosses. J. Anim. Breed. Genet., 120, 363–393.

Goldblum S.E., Ding X., Funk S.E., Sage E.H. (1994): *SPARC* (secreted protein acidic and rich in cysteine) regulates endothelial cell shape and barrier function. Proc. Natl. Acad. Sci. USA, 12, 3448–3452.

Goldoni S., Owens R.T., McQuillan D.J., Shriver Z., Sasisekharan R., Birk D.E., Campbell S., Iozzo R.V. (2004): Biologically active decorin is a monomer in solution. J. Biol. Chem., 20, 6606–6612.

Hellemans J., Mortier G., De Paepe A., Speleman F., Vandesompele J. (2007): qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol., 8, R19.

Horiuchi K., Amizuka N., Takeshita S., Takamatsu H., Katsuura M., Ozawa H., Toyama Y., Bonewald L.F., Kudo A. (1999): Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta. J. Bone Miner. Res., 14, 1239–1249.

Lee S.S., Chen Y., Moran C., Stratil A., Reiner G., Bartenschlager H., Moser G., Geldermann H. (2003): Linkage and QTL mapping for *Sus scrofa* chromosome 5. J. Anim. Breed. Genet., 120, 38–44.

Maguchi M., Nishida W., Kohara K., Kuwano A., Kondo I., Hiwada K.. (1995): Molecular cloning and gene mapping of human basic and acidic calponins. Biochem. Biophys. Res. Commun., 5, 238–244.

Pierzchala M., Kuryl J., Reiner G., Bartenschlager H., Moser G., Geldermann H. (2003): Linkage and QTL mapping for *Sus scrofa* chromosome 16. J. Anim. Breed. Genet., 120, 126–131.

Rehfeldt C., Fiedler I., Dietl G., Ender K. (2000): Myogenesis and postnatal skeletal muscle cell growth as influenced by selection. Livest. Prod. Sci., 66, 177–188.

Stratil A., Knoll A., Horák P., Bílek K., Bechyňová R., Bartenschlager H., Van Poucke M., Peelman L.J., Svobodová K., Geldermann H. (2008): Mapping of the porcine *FBN2*, *YWHAQ*, *CNN3*, *DCN*, *POSTN*, *SPARC*, *RBM39* and *GNAS* genes, expressed in fetal skeletal muscles. Anim. Genet., 39, 204–205.

te Pas M.F.W., de Wit A.A.W., Priem J., Cagnazzo M., Davoli R., Russo V., Pool M.H. (2005): Transcriptome expression profiles in prenatal pigs in relation to myogenesis. J. Muscle Res. Cell. Motil., 26, 157–165.

Vallee M., Beaudry D., Roberge C., Matte J.J., Blouin R., Palin M.F. (2003): Isolation of differentially expressed genes in conceptuses and endometrial tissue of sows in early gestation. Biol. Reprod., 69, 1697–1706.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol., 3, 34 pp.

Wimmers K., Murani E., te Pas M.F., Chang K.C., Davoli R., Merks J.W., Henne H., Muraniova M., da Costa N., Harlizius B., Schellander K., Böll I., Braglia S., de Wit A.A., Cagnazzo M., Fontanesi L., Prins D., Ponsuksili S. (2007): Associations of functional candidate genes derived from gene-expression profiles of prenatal porcine muscle tissue with meat quality and muscle deposition. Anim. Genet., 38, 474–484.

Zhang H., Apfelroth S.D., Hu W., Davis E.C., Sanguineti C., Bonadio J., Mecham R.P., Ramirez F. (1994): Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices. J. Cell Biol., 5, 855–863.

Received: 2007–11–27
Accepted after corrections: 2008–02–25

Corresponding Author

Ing. Karel Bílek, Mendel University of Agriculture and Forestry in Brno, Zemědělská 1, 613 00 Brno, Czech Republic
Tel.: +420 545 133 377, e-mail: kabyk@post.cz