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Transcriptome expression profiles in prenatal pigs in relation to myogenesis

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Abstract

Myogenesis, the formation of muscle fibers, is a complex process. Pigs have been selected for efficient muscle growth for the past decades making them interesting to study myogenesis. We studied expression profiles of genes known to affect myogenesis, muscle structural proteins, and energy metabolism in prenatal pigs from 14 to 91 days of gestation. Primary and secondary muscle fiber formation takes place during days 30–60 and 54–90 of gestation, respectively. Differential expression and expression levels of the genes were studied using microarray technology. Gene activation and repression profiles were studied counting the number of spots with detectable signal. The number of spots for muscle tissue structural protein genes showing upregulated expression increased constantly from day 14 until day 91of gestation indicating continued activation of genes during this period. The mRNA expression level of the genes showed a peak around day 35 of gestation. The expression levels of genes affecting myogenic differentiation (stimulating and inhibiting) showed a peak at day 35 of gestation. The number of spots for differentiation-stimulating genes showing differential expression reaches a first peak around day 35 of gestation and a nadir at day 49 of gestation while the number of spots for differentiation-inhibiting genes reaches a nadir at day 35 of gestation. Myogenic differentiation seems less a matter of the expression level of genes affecting differentiation, but depends on the balance between the number of significantly activated genes for stimulating and inhibiting differentiation. Genes stimulating myoblast proliferation showed a small peak expression prior to day 35 of gestation indicating myoblast proliferation before differentiation. The number of spots and the expression levels of genes for glycolysis and ATP-metabolism are at a nadir around days 35 and 49–63 of gestation suggesting that the energy metabolism is low during fusion of myoblasts into multinucleated muscle fibers.

Introduction

Mammalian myogenesis, the formation of new multinucleated muscle fibers from mononucleated precursor cells called myoblasts, is an exclusive prenatal process determining muscle characteristics such as fiber numbers, which may be related to muscle strength and function (Rehfeldt *et al.*, 2000). Muscle fiber formation takes place in two waves, the primary and secondary muscle fiber formation (Wigmore and Evans, 2002). Each wave consists of proliferation of myoblasts and fusion to form new muscle fibers. While primary muscle fibers form *de novo*, secondary myofibers form using the primary fibers as a template.

Improving growth rate and muscularity has been the primary focus during the past decades in pig breeding (Merks, 2000). This makes pigs a good animal model to study myogenesis. Furthermore, the two waves of muscle fiber formation have relatively long periods of time in the pig, i.e. approximately day 30–60, and day 54–90 of gestation (Wigmore and Stickland, 1983). These long periods – relative to gestation time in laboratory animals – enable the study of myogenesis in detail.

Myogenesis is under complex genetic regulation. The Muscle Regulatory Factors (MRF) gene family are transcription factors activating muscle-specific genes during different stages of myogenesis (Olson,

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1990; Weintraub *et al*., 1991). Myf-5 and MyoD regulate proliferation of myoblasts, myogenin regulates terminal differentiation, and MRF4 is mainly involved in maintenance of muscle tissue (Olson, 1990; Weintraub *et al*., 1991). Genetic influence of the MRF gene family on porcine muscle development has been shown before. Genetic variation in porcine myogenin was related to muscle and general body growth (Soumillion *et al*., 1997; te Pas *et al*., 1999a) while myf-5 did not show such a relationship (te Pas *et al*., 1999b). Furthermore, postnatal expression levels of myf-5, MyoD, and myogenin were related to growth rate (te Pas *et al*., 2000). (See for a review of the myogenesis in livestock te Pas and Soumillion, 2001).

The expression of the MRF genes is under tight temporal and spatial regulation, and numerous factors affecting MRF expression levels are known. A network of genes affects the expression patterns of the MRF genes (Olson, 1993; Rawls and Olson, 1997; Capdevila and Johnson, 2000; Dobosy and Selker, 2001; Kitzmann and Fernandez, 2001; Lee *et al*., 2001; Zhu *et al*., 2001). By doing so they affect muscle and body growth potential. Using microarray technology we studied the porcine expression of genes known to affect myogenesis in laboratory animals and *in vitro* model systems. Microarray technology can simultaneously measure the differential expression of a large number of genes in a given tissue and may identify the genes responsible for different phenotypes. The aims of this study were (1) to describe specific myogenesis-related transcriptome profiles in pigs during prenatal muscle development and (2) to relate these profiles to biological function.

Materials and methods

Animals and collection of tissues

Embryos and fetuses of Duroc pigs were collected at 14, 21, 35, 49, 63, 77, and 91 days of gestation. The sows were slaughtered at a commercial slaughterhouse and the uterus containing the litter was collected immediately after bleeding the animal. Fourteen-day embryos were too small to collect individually. Therefore, each uterus horn was flushed with phosphate buffered saline (PBS, pH7.5). Thus, results obtained with 14 days embryos are pooled embryos of one uterus horn. Although 21-day embryos were collected individually, no muscle tissue was observed and whole embryos were stored. In 35-day embryos a white colored region where longissimus muscle formation was taking place was observed. This region was excised and stored. However, since the muscle itself was not visible yet, the stored tissue may contain some skin and probably other tissues such as adipose tissue as well. The longissimus muscle tissues from fetuses aging 49–91 days of gestation were collected. All isolates were snap frozen in liquid nitrogen and stored at −80°C until use.

Microarray construction

The microarray contained genes with known effects on myogenesis, energy metabolism, and muscle tissue structural genes derived from a literature study (for references and additional information about the genes and the microarray see Table 1 of the additional

Table 1. Primers (A) and probes (B) for real time PCR

A Gene	Primers	EMBL	Location (nt)
GAPDH	Forward: GGCTCTCCAGAACATCATCC	AF017079	938–957
	Reverse: CCCAGCATCAAAGGTAGAAGA		1229–1209
Col3A1	Forward: GCCATCCAGGACAACCAG	AU059332	26–43
	Reverse: ATCGGGACTAATGAGGCTTTC		243–223
EPO-receptor	Forward: GAACCAGCCGCAGATGATG	AF274305	1198–1216
	Reverse: CCAGAGCAGATGAGCAGAAGG		1393–1373
TGF-beta2	Forward: CAACCGGCGGAAGAAG	X70142	123–138
	Reverse: CGTTTTGCCGATGTAGTAGAG		426–406
Beta-catenin	Forward: GACGCTGCTCATCCCAC	AB046171	543–559
	Reverse: CAGCGAGCCGTTTCTACA		838–821
B Gene	Probe-name	EMBL	Location (nt)
GAPDH	FL: CCATGCCAGTGAGCTTCCCG	AF017079	1029–1009
	LC640: GAGCTCAGGGATGACCTTGCCC		1007–986
Col3A1	FL: GCTTTTTCACCTCCAACACCAGCG	AU059332	125–102
	LC640: GGCAGCAGCCCCACCACC		99–82
EPO-receptor	FL: CACAGCCTGGTGGTGATTTGGAC	AF274305	1319–1341
	LC640: GGCGGCCATGGATGAAGCC		1343–1362
TGF-beta2	FL: TAAACCCAGAAGCTTCTGCTTCCCC	X70142	344–368
	LC640: GCTGCGTGTCCCAGGATTTAGAACC		371–395
Beta-catenin	FL: GCTTCCAGACATGCCATCATGCG	AB046171	747–769
	LC640: CTCCTCAGATGGTGTCTGCAATTGTACGT		772–800

The fluorescein group is attached to the 3′-end of the FL probe, the red fluorochrome LC640 is attached to the 5′-end of the LC640 probe and the 3′-end contains a phosphate group.

information). Several of the genes were members of closely related gene families. In such cases a number of family members were also placed on the microarray. These genes were cloned from pig mRNA isolated from the embryos/fetuses. RNA from unrelated embryos/fetuses, two of each age, were isolated, reverse transcribed, and the cDNAs were pooled. Gene-specific primers (see Table 1, additional information) were constructed and used to amplify a fragment on this cDNA pool. The fragments were cloned and if the PCR product did not exactly match the expected length were sequenced to verify the product (see also additional information, Table 1). Furthermore, 309 sequenced clones of an adult-pig-muscle-specific cDNA library (Davoli *et al*., 2001) including many muscle structural and energy metabolism-related genes were placed on the microarray. In total the microarray contained 557 genes with known identity and function.

The clones were amplified in four 50-μl PCR reactions to obtain a total 200 μl total volume PCR product for each clone. These four different PCR products for each clone were collected in the same tube of a 96 well microtiter plate and purified according to the Sephadex protocol. The purified products were precipitated using the absolute ethanol–Sodium acetate–70% ethanol protocol and resuspended in 20 μl spotting buffer (1 M phosphate buffer pH 5.8, 50% DMSO). After that the length, the quantity, and the quality of each fragment was checked on a 1% agarose gel. Two nano liter of the products were spotted on glass slides in duplicate.

Microarray hybridization and analysis

RNA from the six embryos per prenatal age was isolated using the Trizol–Phenol method (Life Technologies, Breda, The Netherlands). Two microgram of RNA for each stage was labeled with Cy3 or Cy5 using the TSA labeling and amplification kit protocol (Perkin Elmer Life Sciences, Inc., Langen, Germany). Hybridizations compared always two prenatal stages of follow up ages of gestation, i.e. 14–21 days, 21–35 days, etc. Hybridizations were performed in duplicate and in duplicate dye swap. Hybridization was done for 16 h at 65°C. After hybridization the slides were rinsed according to the stringency washing protocol recommended by the manufacturer.

Microarrays were scanned using the GeneTac2000 scanner (Genomics Solutions, USA) for 180 s. Each microarray was analyzed independently using the following steps: (1) Normalization of raw scanning data, which included (a) background correction – the background signal was determined using blank spots, and spots with water but no PCR product, (b) normalization (i) using all spots, and (ii) per patch; all intensity dependent using a LOWESS (locally weighted scatterplot smoothing, Cleveland, 1974; Park *et al*., 2003) fit; After this the normalized spots are represented by their M and A values following the procedure as described by Pool *et al*. (2003):

$M = \log_2$ (Cy5/Cy3), Thus the M-value is the ratio between the expression of the gene in the Cy5 and Cy3 labeled sample indicating the differential expression between the Cy5 and Cy3 labeled RNA samples – thus, a positive M-value indicates that the expression of the Cy5 labeled RNA is higher than the expression of the Cy3 labeled RNA, and a negative M-value indicated the reverse situation;

$A = (\log_2 [\text{Cy5} \times \text{Cy3}])/2$, Thus the A-value indicates a weighed mean expression level of the Cy5 and Cy3 labeled RNA samples (Yang *et al*., 2002).

Additionally the significance of the difference (M-value) is indicated by the P-value, two-sided tested on a log–logistic distribution. (2) Next, normalized spots with difference Cy3–Cy5 $P > 0.05$ and/or $-1.58 < M < 1.58$ ($\log_2$ scale) were discarded leaving only those genes with a difference in expression level of 3× or more (normal scale) according to the protocol of the manufacturer of the labeling kit (Perkin Elmer) to eliminate false positive results. (3) The remaining spots were analyzed for up or down regulation of expression by comparing the expression levels in the breeds using the M- and A-values using the Spotfire pro 7 software (BioASP, Amsterdam, The Netherlands). (4) The gene activation and repression profiles were studied by counting the number of spots (genes) with a detectable signal. The results were also analyzed for number of spots with differential expression to investigate the overall effect on biological groups (see also next step). (5) Finally, the results were analyzed with biological interpretation of data using available data on the physiology of the genes, is described in the Results section.

Real time PCR validation

To validate the results of the microarrays five genes of step (4) of the normalization-analysis of the microarrays (see above) were selected and analyzed with real time PCR using the Lightcycler equipment (Roche Diagnostics, Almere, The Netherlands). Genes were chosen in each functional group. Because of the relevance three genes were chosen in the myogenesis affecting group (EPO-receptor, β-catenin, and TGFβ2), one household gene in the energy metabolism group (GAPDH) and a muscle structural gene (collagen 3A1). Primers were designed on the cDNA sequence to amplify a 100–300 bp fragment and probes containing a fluorescein were designed according to the rules set by the manufacturer (Table 1). All reactions had an annealing temperature of 60°C except TGF-beta2 (55°C) and a magnesium concentration of 3 mM except beta-catenin (5 mM). For real time PCR each developmental age was represented by individual RNA samples from the same six embryos or fetuses used in the microarray experiments. Reverse transcription and PCR were done on individual RNAs. A second set of cDNA samples of different embryos/fetuses supplied by Dr K. Wimmers (University of Bonn,

Germany) was used for independent verification of the results.

Normalization of RT-PCR usually uses structural (e.g. beta-actin) or household genes (e.g. GAPDH), several of which were included in the microarrays. The results of the microarrays indicated that the expressions of these genes were regulated during the investigated prenatal period. Thus these genes were not suitable for normalization (Radonić *et al.*, 2004). Therefore, we used the 18S rRNA expression as an independent non-protein gene for normalization. The 18S rRNA expression showed no differential expression during the 35–91 days of gestation period, while being low in embryos aged 14-days and 21-days. This may relate to the difference of the isolated tissues.

Results

Microarray analysis

Prenatal age dependent development of expression pattern

Based on known biological function genes were grouped into three major groups: myogenesis (n = 178), energy metabolism (n = 80), and muscle structural (n = 48) genes. The first two groups have been sub-divided into pathway-specific subgroups (Table 2). Some genes belong to more than one group. Results were analyzed for (1) up/down regulation – i.e. the ratio between the expression level in one age prenatal samples versus another age prenatal samples, (2) for general expression level, and (3) for gene activation/repression profiles. Table 2 shows that most, but not all genes that belong to a (sub) group show differential expression, and that not all genes showing differential expression do so at all prenatal ages.

Expression level profile

The expression level of the genes was studied using the A-values of spots. The profiles (Figure 1(a)) indicate that the genes involved in muscle fiber formation, i.e. differentiation-stimulating, differentiation-inhibiting, and muscle fiber structural genes, show a peak expression around day 35. The proliferation-stimulating groups of genes show lower peak levels. A detailed analysis (Figure 1(b)) shows that proliferation-stimulating genes show a much smaller peak at the same time. Furthermore, expression of glycolysis metabolism genes is at a nadir at the two period's central in differentiation: around days 35 and 49–63. ATP metabolism follows that profile later in time while oxidative phosphorylation has less variable expression.

Gene activation and repression profiles

Activation and repression of gene expression are important characteristics of prenatal development. Therefore we studied these processes counting the number of spots on the microarrays with detectable expression levels, i.e. spots with expression levels above background levels + SD of the background. M- and A-values were calculated for these spots only. Next the numbers of spots involved with differential expression were analyzed. Figure 2(a) shows that the number of spots of muscle fiber structural genes rapidly increases from day 21 until day 49. While still spots indicate increased expression of genes until day 91 the genes induced early (day 21) show reduced expression from day

Table 2. Number of genes within each functional group and number of genes showing differential expression within each group and each prenatal age

Biological functional pathway	Day by day comparison							
	n	*n*-list	14–21d	21–35d	35–49d	49–63d	63–77d	77–91d
Energy metabolism genes	80	74						
Glycolysis	22	21	13	17	14	7	13	10
Oxidative Phosphorylation	17	17	5	14	10	4	7	7
ATP metabolism	18	17	15	15	9	7	4	6
Fatty acid metabolism	10	9	4	2	4	1	5	2
Miscellaneous*	9	9	3	5	6	3	4	2
Myogenesis genes	178	173						
Differentiation-stimulating	48	48	15	18	21	16	18	20
Differentiation-inhibiting	44	44	20	20	24	13	13	11
Proliferation stimulating	42	40	16	19	18	9	14	11
Proliferation inhibiting	16	16	6	7	7	2	8	5
Migration	5	5	3	2	2	3	0	0
Structural genes	48	45						
Myocyte structural genes	48	45	14	27	24	17	25	16
Early structural genes	8	8	6	6	2	4	4	4

Groups of genes involved in energy metabolism, myogenesis, and muscle tissue structure were created. Groups are divided in subgroups indicating pathways. n: the total number of genes within on the microarray; n-list: the number of genes giving at least at one prenatal age significant differential expression.

* Containing genes such as for Creatine metabolism, being important for energy metabolism but not related to groups indicated above.

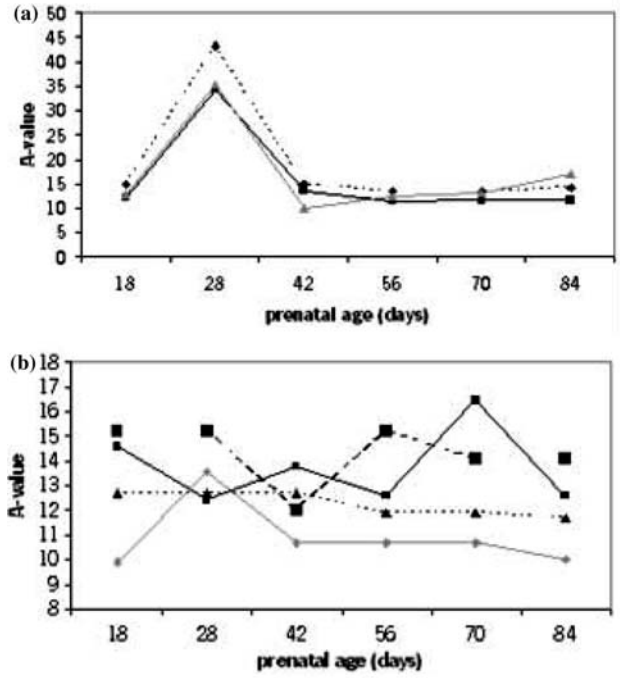

Fig. 1. Profile of mean *A*-values per group of genes indicating absolute expression levels per group on each developmental age. (a) Black line: differentiation-stimulating genes; gray line: differentiation-inhibiting genes; broken line: Structural genes (b) Black line: glycolysis genes; gray line: proliferation-stimulating genes; broken line: ATP-metabolism; dotted line: oxidative phosphorylation. Please note the different *Y*-axis scale between figures (a) and (b).

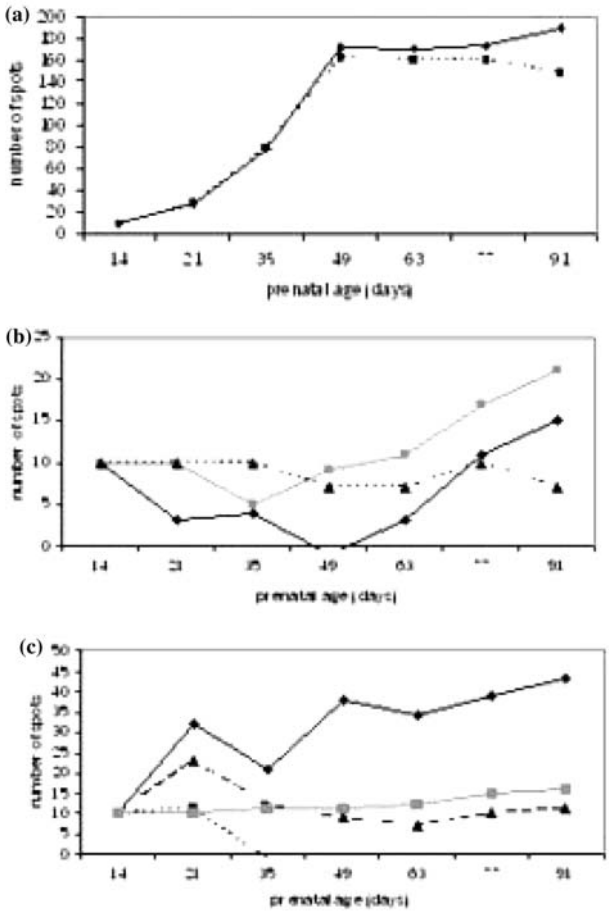

Fig. 2. Gene activation and repression profiles as measured by the number of spots with detectable signal of each prenatal developmental age. (a) Structural genes, black line: structural genes; broken line: early structural genes, (b) myogenesis affecting genes, black line: differentiation-stimulating genes; gray line: differentiation-inhibiting genes; broken line: proliferation-stimulating genes; and (C) Energy metabolism genes, black line glycolysis genes; gray line: fatty acid metabolism genes; broken line: ATP-metabolism genes; dotted line: oxidative phosphorylation genes.

49 onwards (Figure 2(a)). Analysis of groups of myogenesis involved genes (Figure 2(b)) indicate that the number of genes showing differential expression involved in stimulation of proliferation is relatively stable, showing a small decrease between days 35 and 63 of gestation, and a second small decrease towards the end of gestation. Differentiation-stimulating gene numbers are reduced initially, but gene numbers rise towards days 35 and days 63–77. The numbers of differentiation-inhibiting genes showing differential expression is reduced around day 35 and, to a lesser extend, around day 63. Changes in the number of differentially expressed energy metabolism genes (Figure 2(c)) indicate that the number of genes involved in glycolysis and ATP metabolism increase between days 14 and 21 and are reduced at day 35. After this the number of differentially expressed glycolysis genes is increased until day 49, is reduced again at day 63, and increases afterwards, while the number of genes involved in ATP metabolism is relatively stable. The number of genes involved in oxidative phosphorylation showing differential expression seems to be reduced until day 49 of gestation and remains stable afterwards.

Real time PCR validation of microarray results

Real time PCR validation of microarrays was performed with five genes differentially expressed and chosen such that all major groups of genes analyzed are represented (Figure 3). Col 3A1 represents the (early) muscle structural genes, GAPDH represents energy metabolism genes, especially glycolysis metabolism, EPO-receptor, beta-catenin, and TGF-beta2 represent myogenesis genes, differentiation-inhibiting and proliferation-stimulating, proliferation-stimulating, and differentiation-inhibiting, respectively. The real time PCR analyses were done on the individual RNA isolates (not pools as used for the microarrays). The standard error (SE) was variable with the prenatal age. The SE may be up to 30% of the mean at 14 days of age (mainly because the RT-PCR failed on a few samples) while at older ages the SE was 1–5% of the mean (data not shown).

The Col3A1 mRNA expression is barely detectable at 14 days of age, is upregulated at 21 days of age reaches a maximum at 35 days. Col3A1 is down regulated after 35 days of age, with a small second peak in the expression around days 63–77 of age. This correlates with the myogenic differentiation representing

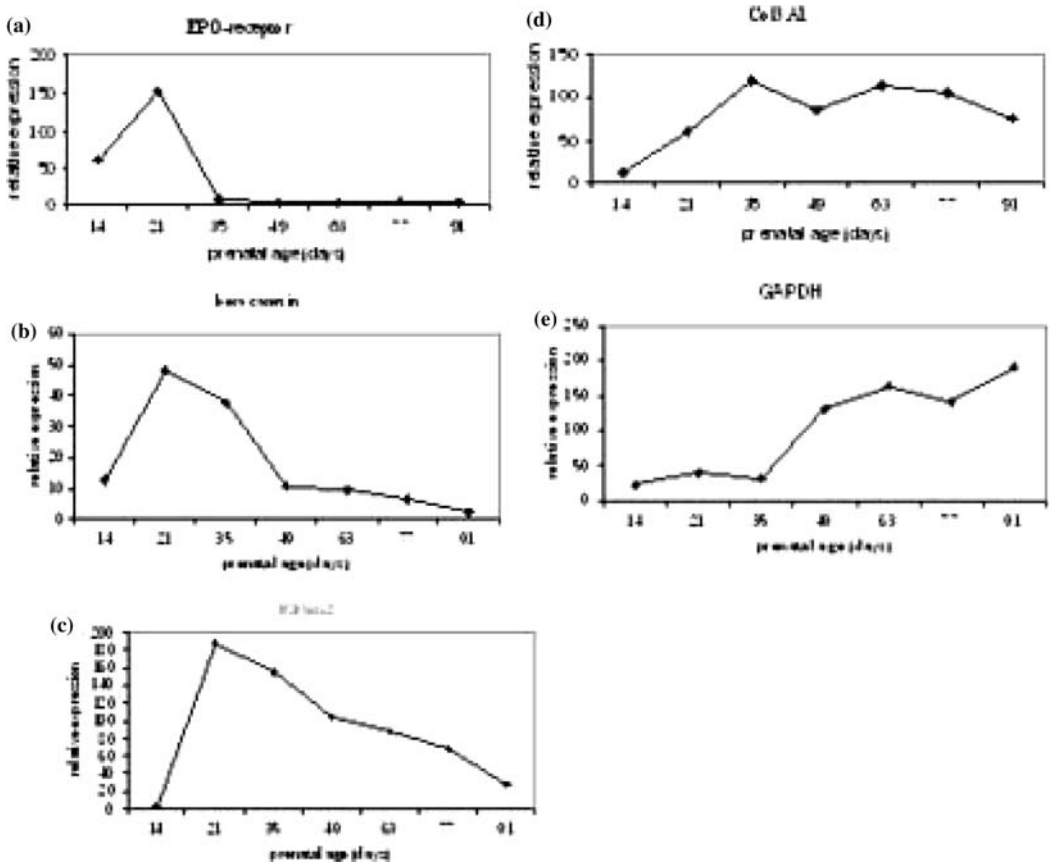

Fig. 3. Real time PCR of five genes representing the major functional groups. Erythropoietin (EPO)-receptor (a), beta-catenin (b), and Transforming Growth Factor (TGF)-beta 2 (c) represent myogenic genes, differentiation-inhibiting and proliferation-stimulating, proliferation-stimulating, and differentiation-inhibiting, respectively.Collagen (Col) 3A1 (d) represents the (early) muscle structural genes, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (e) represents energy metabolism genes, especially glycolysis metabolism. The data were normalized against the 18S rRNA expression.

genes indicating peak expression at 21 days of age (EPO-receptor and TGF-beta2) or 21–35 days of age (beta-catenin). Meanwhile, the GAPDH gene representing energy metabolism is at a nadir around day 35 of gestation. After the nadir its expression level increases, but reaching a second smaller nadir at day 77 of gestation. These results were confirmed with independently isolated prenatal samples of different animals including different litters (data not shown).

Discussion

Microarray analysis of prenatal muscle tissue formation

Microarray analysis is a very powerful tool allowing the analysis of complete transcriptomes of tissues. The combined expression analysis of all or many genes from pathways may provide insight into the combined regulation of processes – such as the formation of tissues – or pathways and may help to discover how various pathways are connected. If many genes that regulate a process are known this process can be studied in detail by producing specialized dedicated microarrays containing especially those genes.

However, the enormous amount of data generated by microarray analysis requires stringent statistical evaluation to avoid the generation of many false positive results. Also the labeling and hybridization methods used have limitations. In our study we therefore have first eliminated all data with $P > 0.05$, and thereafter all data with M-values indicating a differential expression smaller than three times – as recommended to eliminate false positive results. As a consequence many data are regarded non-significant, among them will be false negative data. Although on the individual gene level the differences are big (many genes were not included in the second analysis) the conclusions for the functional groups are similar. Group variability was reduced in the second analysis compared to the first analysis, probably caused by false positives. Finally, these false negative results of the microarray analysis may result in small differences with analysis by other methods such as real time PCR, which are able to indicate smaller differences too.

Early embryo sampling

Very young embryos are small. It is impossible to isolate 14 day old individual pig embryos on a large

enough scale fast enough to avoid RNA degradation and/or change of RNA expression of genes. Above that, the amount of RNA isolated from these embryos would be far too small to be used in microarray experiments. Therefore we have collected 14-day embryos flushing an entire uterus horn. As a consequence these samples contain both embryonic and extra-embryonic prenatal cells. For 21-days old embryo samples contain individual whole embryos. These embryos are too small to isolate and use individual tissues, and muscle tissue is not visible in these young embryos. Therefore, the results obtained with these young embryos may be partly influenced by other tissues. This may be particularly the case for energy metabolism genes. Likewise some signal from non-muscle specific transcripts of myogenesis and structural genes cannot be excluded in these young embryos. On the other hand, at best muscle precursor cells will be present in these embryos, mainly in somites. We have considered this by including eight genes known to affect somite development into the group of myogenesis affecting genes (see Table 1, additional information). None of these genes show significant differential expression at any developmental stage (data not shown). Therefore, we consider the data obtained with these samples reliable. Finally, to a minor extend also the samples of 35-day old embryos differ. These samples contain mainly the area where muscle formation is taking place (but no muscle tissue is visible yet). Some adipocytes and skin tissue may be in the samples. However, adipocytes constitute a part of normal muscle tissue too. Nevertheless, it is good to recognize that only from day 49 of gestation and onwards pure longissimus tissue is sampled and analyzed.

Prenatal muscle-specific expression profile related to myogenesis

Prenatal development is characterized by massive changes at the cellular phenotypic and the gene expression levels during the formation of body tissues. Muscle tissue is a major constituent of the body and muscle fibers are the major constituent of muscle tissue. Therefore we studied the expression profiles of genes known to be involved in muscle fiber formation and muscle structural genes. Furthermore, differentiation requires massive cell phenotype rebuilding which include expression of muscle developmental stage and phenotype-related proteins. In the case of muscle cells this also includes fusion of myoblasts and restructuring of the resulting multinuclear cell into a functional muscle fiber. The energy metabolism should be suitable to supply the tissue with the required energy for the processes including the protein specific synthesis of each developmental step. Therefore, we included genes representing glycolysis, oxidative phosphorylation, and ATP metabolism in our microarray.

Our results indicate firstly that the changes in myogenesis related, muscle fiber structural, and energy metabolism-related genes are more intense in the period of primary muscle fiber formation than in the period of secondary muscle fiber formation. We observed that at the moment of muscle fiber formation the expression of differentiation-stimulating genes, differentiation-inhibiting genes and muscle fiber structural genes is increased.

When differentiation of muscle fibers takes place (i.e. day 35 of gestation for primary muscle fiber formation) it is remarkable that the number of muscle structural genes and muscle differentiating genes showing differential expression remains largely unchanged (Table 2) while the number of spots for these genes increases (Figure 2). At the same time number of differentiation-inhibiting genes showing differential expression is similarly unchanged (Table 2) while the number of spots is reduced (Figure 2). All three groups of genes show a high expression level (*A*-value) at the moment of differentiation (Figure 1). Each gene is represented on each time point by a maximum of eight spots (duplicate spots per microarray, four microarray hybridizations per time point). Only spots indicating a reliable significant differential expression are counted. The number of spots per gene is thus a measure for the repeatability of the difference of the expression levels, which may indicate the reliability of the measurements (the more significant spots, the higher the repeatability of the result, the more reliable the data). Alternatively, when the number of significant spots per gene decreases this may just indicate a lesser degree of difference in expression inducing false negative results. The interpretation of the above mentioned changes in number of spots per gene for the differentiation-stimulating and the muscle structural genes may indicate increased activity of these genes while the opposite is true for the myogenesis inhibiting group of genes. Thus, differentiation seems to be the result of a balance between the number of (spots) genes stimulating and inhibiting differentiation rather than the expression level of the expressed genes themselves. If the expression level of a higher number of (spots) genes stimulating differentiation increases and the number of genes inhibiting differentiation decreases – despite a higher expression level of the remaining genes – differentiation takes place. Dedieu *et al.* (2002) proposed that initiation of the fusion step – which is an essential component of differentiation, only proceed when the MRF genes reach a threshold. Our results indicate that this threshold may not only depend on the expression levels of the MRF genes but requires the balance of many differentiation-stimulating and -inhibiting genes.

As formed myofibers require specific structural genes it is not surprising to note that these genes also show increased expression. Within the muscle fiber structural genes there seem to be two components: Those that are expressed early (from day 21 onwards) and those that are expressed later (after day 35) with the first showing decreased expression after day 49 (but with a small peak during secondary muscle fiber

formation) and the second group remaining highly expressed, at least until day 91 when muscle fiber formation is completed.

Pigs have a history of selection for increased muscularity. Our data indicate that day 35 of gestation, and to a lesser extent also the period around day 63 of gestation are major moments in myogenesis. It would be interesting in future studies to investigate the expression profiles at these moments of prenatal development in more detail. It can be assumed that many processes regulating postnatal muscularity and muscle failure can be explained from such studies.

Myogenesis is regulated by the MRF genes, a family of muscle fiber-specific transcription factors that regulate developmental-specific gene expression (Olson, 1990, Weintraub *et al*., 1991), which has been shown to be active in pigs too (for a review see te Pas and Soumillion, 2001). Many proteins have been shown to affect myogenesis via the MRF genes (for many references see Table 1, additional information). However, in our microarray analysis where many of the above mentioned genes showed indeed differential expression we did not find reliable (i.e. repeatable significant according to the specifications of the labeling kit) differential expression of the MRF genes. This may be caused by the analysis of the microarray as discussed above taking only differential expression greater than three times as reliable. More subtle changes in the expression are regarded as not significant. However, also in our analysis taking all data with $P < 0.05$ into account the MRF genes showed only marginally differential expression profiles with sometimes conflicting data. Thus, we consider the expressional changes of the MRF genes as marginal or not important. This does not imply that the MRF genes are not important in pig muscle development, but mRNA expressional changes related to primary of secondary muscle fiber formation are not observed as a major effect in our pigs. Alternatively, several proteins regulate MRF function by binding to the MRF proteins either activating or inhibiting MRF function. Such effects will not be measured with microarray analysis. Thus, the activity of the MRF proteins may be regulated without (big) alterations of mRNA expression.

It has been shown *in vitro* that Insulin-like growth factor (IGF)-I increases myogenin expression 60-fold (Florini *et al*., 1991). While the IGF proteins are also potent prenatal activators, it is mainly the IGF-II that acts prenatal (Rappolee et al., 1992; Stylianopoulou *et al*., 1988). However, neither IGF-I nor IGF-II, nor the receptors were greatly differentially regulated. Thus, in our experiment we may conclude that MRF gene expression seems not regulated by the IGF system. Alternatively, Coutinho *et al*. (1993) showed in a quail selection experiment that increased numbers of muscle fibers were related to delayed expression of MRF genes rather than upregulated expression. It would be interesting to investigate the expression profiles of all myogenesis affecting genes on selection-related timing of expression.

We have shown previously that the expression of the MRF genes was upregulated in post-natal muscle tissue due to selection for increased growth rate and muscle growth (te Pas *et al*., 2000). Similar upregulation may have occurred in the prenatal pig. Since selection-related changes in prenatal expression profiles are unknown we can only speculate on this point. Future research should aim on this as it marks important points in time in the development of body tissues.

Prenatal muscle-specific energy metabolism expression profile related to myogenesis

The results of the energy metabolism were unexpected. Indeed, there seems to be a relationship with the muscle fiber formation, but a negative relationship. As muscle fiber formation takes place, expression of energy metabolism genes is low. Although we realize that microarrays study the transcriptome of cells, which is not necessarily equal to the proteome or the activity of enzymes, it may indicate that differentiation takes place during low energy status of the cells. Energy content of satellite cell cultures has been related to hypertrophy of muscle fibers *in vitro* (Louis *et al*., 2004). Adding creatine to satellite cell cultures increases IGF-I level and expression of the four MRF genes, although only myogenin was increased over three-fold. The authors did not report changes to myofiber numbers, so these results cannot be compared directly, but this is an additional indication for a connection between the myogenesis pathway and the energy metabolism pathways. Furthermore, two recent publications showed regulation of genes involved in energy metabolism during myogenesis. Riera *et al*. (2003) showed down regulation of 6-phosphofructo-2-kinase – a key enzyme in the control of glycolysis and gluconeogenesis – by ubiquitin/proteasome proteolysis during myogenic differentiation in C2C12 cells and Chen *et al*. (2003) showed that BMP9 regulates both phosphoenolpyruvate carboxykinase (PEPCK) – a key enzyme in glycolysis – and Akt kinase – involved in differentiated myotubes. These results, although fragmentary, point to a connection between energy metabolism and myogenic differentiation, as we have shown on a much larger scale.

At present no good biological reasons for this observed effect can be given. It may be suggested that fusion of cells requires low energy status, but our understanding of the role of energy metabolism in myogenesis remains poor.

Summarizing, we have shown that porcine myogenesis is associated with balanced coordinated regulation of expression. This involves genes related to myogenesis (both positively and negatively regulating myogenesis), muscle structural genes, and energy metabolism genes.

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