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Muscle transcriptomes of Duroc and Pietrain pig breeds during prenatal formation of skeletal muscle tissue using microarray technology

Abstract

Mammalian myogenesis is an exclusive prenatal process regulated by the muscle regulatory factor gene family, which itself is regulated by numerous other genes. We developed a microarray consisting of the clones of two muscle-specific cDNA libraries with the addition of 500 genes with known function in myogenesis and energy metabolism. Tissue samples were collected of Duroc and Pietrain prenatal litters of 14 and 21 days of age (complete embryos) and 35, 49, 63, 77, and 91 days of age (longissimus muscle tissue) and RNA was isolated. Microarrays were hybridised with pools of six RNA samples. For each age comparisons between Duroc and Pietrain breeds were made, and transcriptome profile changes in time were made for Duroc pigs.

Comparison of Duroc and Pietrain prenatal muscle transcriptome expression profiles revealed differences in myogenesis regulating genes, suggesting differential timing of myogenesis between the two pig breeds. The differential development of the expression of the muscle structural genes strengthens this conclusion. Furthermore, differences in the expression of the energy metabolism genes were found. The results also suggest that the differential fat content between the Duroc and Pietrain pig breeds already starts to develop during early prenatal development.

The changes in the muscle transcriptome expression profiles during Duroc prenatal muscle development shows a profile of waves of expression of (i) myoblast proliferation stimulating genes,(ii) followed by myoblast proliferation inhibiting and differentiation stimulating genes during the primary muscle fibre development, which is repeated with lower magnitude during secondary muscle fibre development. Furthermore, expression of energy metabolism genes reaches a nadir when differentiation of myoblasts into myotubes takes place.

Microarray expression profiles were validated with five genes showing differential expression in the Duroc – Pietrain comparison, and in the Duroc development in time studies using 18S rRNA for normalisation. The real time PCR confirmed the microarray results.

Key Words: pig breeds, prenatal development, myogenesis, transcriptome profile, differential expression

Zusammenfassung

Titel der Arbeit: **Analyse des Muskeltranskriptoms während der pränatalen Entwicklung des Skelettmuskels bei den Rassen Duroc und Pietrain mittels Mikroarray Technologie**

Die Myogenese beim Säugetier ist ein pränataler Prozess, der durch die Mitglieder der Genfamilie der Muskel regulierenden Faktoren gesteuert wird, die selbst durch zahlreiche andere Gene reguliert werden. Ein Microarray bestehend aus Klonen von zwei Muskel-spezifischen DNA Bibliotheken sowie 500 Genen mit bekannter Funktion in der Myogenese und dem Energiehaushalt wurde konstruiert. Fetale Gewebeproben wurden bei den Rassen Duroc und Pietrain gesammelt (Tag 14 und 21 komplette Embryonen; Tag 35, 49, 63, 77 und 91 Gewebe des Musculus longissimus dorsi) und RNA wurde isoliert. Mikroarrays wurden mit Pools von je sechs RNA-Proben hybridisiert. Für jeden Zeitpunkt der pränatalen Entwicklung wurden Vergleiche zwischen den Rassen vorgenommen und für die Rasse Duroc Vergleiche zwischen den Entwicklungszeitpunkten.Unterschieden in der Expression der Myogenese-steuernden Gene zwischen Duroc und Pietrain weisen auf einen unterschiedlichen zeitlichen Verlauf der Myogenese bei den beiden Rassen hin. Unterschiede in der Expression von Muskelstrukturgenen bestätigen dies. Auch Differenzen in der Expression von Genen des Energiehaushalts wurden gefunden. Die Ergebnisse deuten zudem darauf hin, dass sich Unterschiede im Fettgehalt bei den Rassen Duroc und Pietrain bereits während er frühen pränatalen Entwicklung herausbilden. Die Änderungen der Genexpression während der pränatalen Muskelentwicklung bei der Rasse Duroc erfolgt in Wellen mit während der Bildung der primären Muskelfaser (i) zunächst Genen, die die Myoblastenproliferation stimulieren (ii) gefolgt von Genen, die die Myoblastenproliferation hemmen und ihre Differenzierung fördern und (iii) Wiederholung dieser Abfolge während der Bildung der sekundären Muskelfasern. Die Expression von Genen des Energiestoffwechsel ist minimal in den Phasen der Differenzierung. Die Ergebnisse der Mikroarray-Analyse wurden für fünf Genen mittel Echtzeit-PCR bestätigt.

Schlüsselwörter: Schwein, pränatale Entwicklung, Myogenese, Transkriptom, Expressionsprofil, Differentielle Expression

Introduction

The formation of muscle fibres (myogenesis) from precursor cells (myoblasts) is an exclusive prenatal process in mammals (REHFELDT et al., 2000). Myogenesis proceeds in two highly regulated waves following the specification and amplification of myoblasts in somites. During the primary wave myofibres are formed de novo from myoblasts while secondary myofibres are formed using the primary myofibres as a template (WIGMORE and STICKLAND, 1983).

During the last decades pigs have been selected for increased skeletal muscle mass on their carcasses (meat percentage). Skeletal muscle mass mainly relates to muscle fibre numbers (hyperplasia) and thickness (hypertrophy). While hypertrophic growth is mainly taking place postnatal, hyperplastic growth is exclusive prenatal. Pig breeds differ for myofibre hyperplasia, hypertrophy, and myofibre typing. Pig muscle composition is of high economic importance. Thus, the study of the prenatal myogenesis may highlight important fundamental processes that can be used in pig breeding to improve pig meat production. In the pig primary muscle fibre formation takes place in a period around day 35, and secondary muscle fibre formation is taking place in a period at approximately day 65 of gestation (WIGMORE and STICKLAND, 1983).

The muscle regulatory factors (MRF) gene family takes a central position in the regulation of myogenesis (WEINTRAUB et al., 1991). The MRF gene family consists of four genes, with MyoD and myf-5 expressed during the proliferation of myoblasts, myogenin expressed during the differentiation (i.e. fusion of myoblasts to form multi-nucleated myofibres) and with MRF4 mainly expressed in the muscle fibres to maintain their differentiated status (WEINTRAUB et al., 1991). The MRF genes are transcription factors regulating the transcriptome of the myoblasts to induce proliferation or fusion. However, their correct spatial and temporal expression requires strict regulation of their expression too. Over the last decade several proteins and pathways have been discovered involved in the correct regulation of the expression patterns of the MRF genes. Using microarray technology we studied both the expression patterns of the genes involved in the regulation of the MRF genes, and the muscle cell (myoblasts and myofibres) transcriptome profiles during pig prenatal development. Here we report on the transcriptome profiles of the genes regulating the expression of the MRF genes and expression patterns of known muscle structural genes.

Materials and Methods

Animal samples

Duroc and Pietrain prenatal samples were isolated from slaughtering pregnant sows at 14, 21, 35, 49, 63, 77, and 91 days of gestation. Fourteen days embryos were recovered by flushing the uterus horns, and thus consist of a pool of all embryos in a single uterus horn. Although 21 day embryos could be collected individually they were too small to isolate the area where myogenesis took place. For 35 days embryos this area was isolated but muscle tissue was not morphologically recognizable. For all

older foetuses longissimus muscle tissue was collected. Samples were frozen in liquid nitrogen and stored at -80^{0}C.

Microarray construction
Two cDNA libraries were constructed from postnatal pig muscle tissue and placed on a microarray (DAVOLI et al., 2002). A literature survey was done to find the genes known to be involved in the regulation of the MRF genes. These pig genes were cloned from a mixture of RNA isolates from all prenatal stages described above and added to the microarray.

Microarray hybridisation and analyses
The microarrays were hybridised with pools of RNA isolated from the prenatal pig samples. For each breed and prenatal stage RNA was isolated from six samples and pooled. Two microgram of RNA pools was labelled with either Cy3 or Cy5. Microarray hybridisations were done in two different experimental designs. Experiment 1 aims to compare the transcriptome of Duroc and Pietrain breeds at seven prenatal age stages related to muscle tissue formation, and experiment 2 aims to highlight the changes in the transcriptomes during prenatal development related to muscle tissue formation (in Duroc pigs).
Experiment 1
Pools of RNA samples derived from Duroc and Pietrain pigs of equal prenatal age were hybridised to the microarrays. Thus, at seven prenatal ages Duroc-Pietrain breed-comparisons were made.
Experiment 2
Pools of RNA samples of Duroc were hybridised to the microarray in a prenatal age-comparison experiment. The following comparisons of transcriptome expression patterns were done: 14 vs 21, 21 vs 35, 35 vs 49, 49 vs 63, 63 vs 77, and 77 vs 91 days of age. Thus, a total of six different age comparisons were made for Duroc.
All hybridisations were done in duplicate and in dye swap duplicate. Thus, each hybridisation analysis contained four independent hybridisations. Hybridisations were first normalised according to a LOWESS fit protocol followed by analyses using the spotfire software. Shortly, differential expression of genes was recorded, and clustering analysis was used to indicate myogenic pathways. Up or down regulation of a number of genes was verified using real time PCR (data not shown).

Results

Experiment 1
Genes were grouped in two functional groups: Myogenesis related and Energy metabolism (Table). The energy metabolism group was added to the microarray since a literature survey suggested that the level of energy supply was related to myogenesis. Each group was divided into subgroups according to their reported role in myogenesis or energy metabolism. The results describe the expression profile of (the majority of) the genes within each subgroup. Individual genes may differ from this pattern, but these differences are not described in detail here.
Comparison of the differential expression profiles between Duroc and Pietrain pigs revealed that the expression of the genes related to proliferation and differentiation of myoblasts in young embryos (i.e. 14-49 days prenatal age) were higher expressed in

Duroc than in Pietrain embryos, while the opposite was found in older foetuses. This transcriptome profile is supported by the expression profile of the muscle structural genes. These results suggest that myogenesis start earlier in Duroc embryos than in Pietrain embryos. However, at later stage Pietrain foetuses catch up. Alternatively, the results may suggest that myogenesis start at a higher rate in Duroc embryos while in older embryos myogenesis is slowing down. The opposite is for Pietrain where myogenesis start-up slowly but accelerate toward the end of gestation.
The energy metabolism genes show a different profile. With the exception of fatty acid metabolism the energy metabolism genes are expressed to a higher level in Pietrain compared to Duroc. Only at day 35 the situation is reversed. The fatty acid metabolism genes show that fatty acid metabolism start early in Duroc at a higher level than in Pietrain. After day 49 of gestation the expression level in Pietrain foetuses is increased to a higher level than the expression level in Duroc foetuses.

Table
Differential expression between two pig breeds, Duroc and Pietrain, at seven prenatal ages. The genes are grouped according to their known role in myogenesis or energy metabolism. The expression differences are indicated as the ratio of the expression in Duroc and in Pietrain for (the majority of) the genes in the group.

		Duroc : Pietrain ratio						
	N	14d	21d	35d	49d	63d	77d	91d
Myogenesis affecting	**175**							
Differentiation stimulating		~	>	>	>	<	<	<
Differentiation inhibiting		>	>	>	~	>	>	>
Proliferation stimulating		~	>	>	~	~	<	~
Proliferation inhibiting		not enough information *						
Diff / Prol affecting		~	<	<	~	<	>	>
Migration regulating		>	>	>	~	~	~	>
Muscle structural		>	>	>	>	<	<	<
Energy metabolism genes	**61**							
ATP metabolism		<	<	>	<	<	<	<
Oxidative phosphorylation		<	<	>	<	<	<	<
Glycolysis		<	<	>	<	<	<	<
Fatty acid metabolism		>	>	>	>	<	<	<
Miscellaneous		<	<	>	<	<	<	<

~: The genes within the group show similar expression levels in Duroc and Pietrain; >: The mean expression of the genes within the group is higher in Duroc than in Pietrain samples; <: The mean expression of the genes within the group is lower than in Duroc in Pietrain samples; *: The number of genes showing reliable (differential) expression is too low to enable a reliable conclusion.

Experiment 2

The genes were grouped in the same groups as described in Experiment 1. The results were analysed for (1) profile of changes of expression of groups of genes, and (2) activation and silencing of groups of genes.

Profile of the changes of expression of groups of genes

The expression levels of the myogenesis related genes shows that differentiation related genes and muscle structural genes peak around day 35 of gestation (Fig. 1). The myoblast proliferation stimulating genes peak just slightly earlier. While the energy metabolism subgroups - represented by the glycolysis genes - have a much more complicated pattern there seems to be a tendency to have a reduced expression level when differentiation affecting genes are at a peak level.

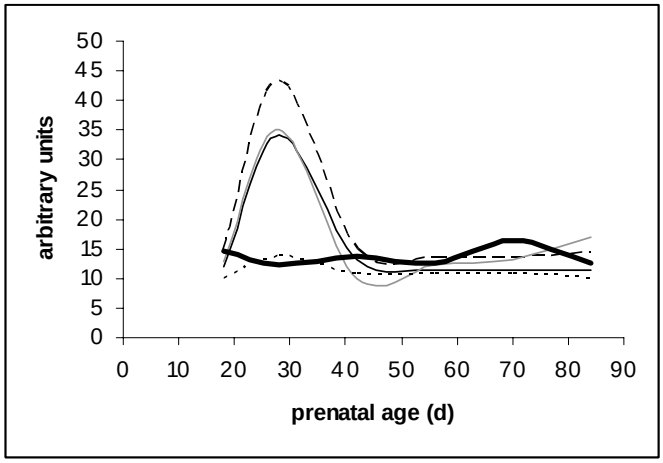

Fig. 1: Profile of mean expression levels per group of genes each developmental age. Black line: differentiation stimulating genes; gray line: differentiation inhibiting genes; broken line: Structural genes; dotted line: proliferation stimulating genes; thick line: glycolysis genes.

Activation and silencing of groups of genes

As already suggested by the results of experiment 1 some of the muscle structural genes are activated early – starting already from day 14 of gestation - and both the number of genes activated and the expression level of the individual genes are increasing until the end of the experiment at gestation time 91 days with a sharp increase in the period 35-49 days of gestation (data not shown). Also many of the myogenesis affecting genes are already activated at day 14 of gestation. The numbers of active genes vary with time, e.g., while differentiation stimulating genes are most activated around day 35 and day 64 of gestation the differentiation inhibiting genes are at a nadir at those times (Fig. 2). The energy metabolism genes represented by the glycolyses genes at a nadir at the time when the number of differentiation stimulating genes is at a peak level.

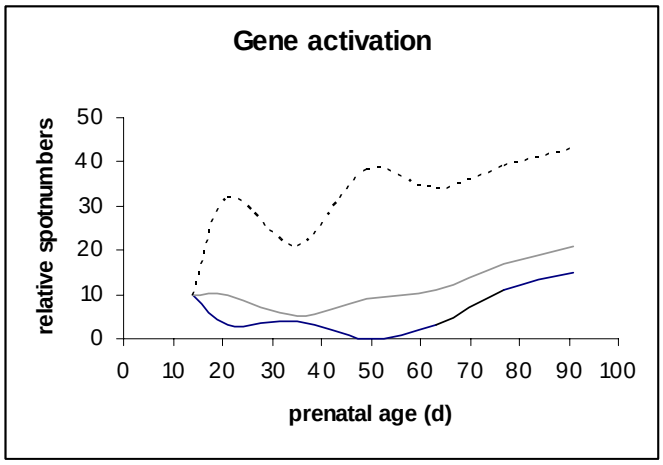

Fig. 2: Activation of genes: analysis of numbers of spots with differential expression of each prenatal developmental age. Black line: differentiation stimulating genes; gray line: differentiation inhibiting genes; broken line: glycolysis genes.

Discussion

Western pigs are selected for high muscle mass during the last decades (MERKS, 2000). Different pig breeds differ in muscle mass and muscle fibre composition. The

Pietrain pig breed with its extremely high muscularity shows predominantly white muscle fibre type, while the Duroc breed is les extreme in muscularity and has more red muscle fibres (SELLIER, 1998). The molecular background of these selection differences remains largely unknown. Muscle mass relates to muscle fibre type, muscle hypertrophy, and to a large extend to muscle fibre numbers. Since muscle fibre formation is an exclusive prenatal event in mammals the study of the transcriptome differences and transcriptome profiles may elucidate the mechanism(s) of past selection response, and may highlight possible future breeding directions. Therefore we studied these processes using microarray technology. The results indicate that indeed there exist remarkable transcriptome differences between Duroc and Pietrain muscle fibre formation profiles. The results suggest that the muscle fibre formation either starts late or begins at a slow speed in Pietrain compared to Duroc. It has been suggested that a characteristic of muscle fibre hyperplasia in selection lines of quails is delayed muscle fibre formation due to an enlarged myoblast proliferation period (COUTINHO et al., 1993). Our results may indicate that a similar mechanism is active in the pig too.

Changes in the level of expression and number of activated genes were studied during myogenesis. The results indicate that the number of activated genes appears to be crucial for the processes taking place. When the number of active genes involved in differentiation induction increases and simultaneously the number of active genes involved in differentiation inhibition decreases differentiation seems to take place. The number of genes involved may suggest that activation of differentiation requires the activation of several pathways. Since it is known that prenatal tissue formation is a highly regulated (spatial and temporal) event this is not surprising.

Energy levels have been related to muscle fibre formation as well (RIERA et al., 2003). Our results in both experiments indicate that the moment of differentiation seems to be associated with a dramatic change in the expression of energy metabolism genes. In experiment 1 the ratio of the expression of energy metabolism genes between Duroc and Pietrain changes abruptly at day 35 of gestation. In experiment 2 we found at days 35 and 63 decreased expressions of the energy metabolism genes. Thus, our results agree with this and indicate that differentiation is associated with a low energy metabolism gene expression. The importance of this remains unknown and further investigation is required on this point.

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References

REHFELDT, C.; FIEDLER, I.; DIETL, G.; ENDER, K.:
Myogenesis and postnatal skeletal muscle cell growth as influenced by selection. Livestock Production Science **66**(2000), 177-188.

WIGMORE, P.M.C.; STICKLAND, N.C.:
Muscle development in large and small pig fetuses. Journal of Anatomy **137** (1983), 235-245.

WEINTRAUB, H.; DAVIS, R.; TAPSCOTT, S.; THAYER, M.; KRAUSE, M.; BENEZRA, R.; BLACKWEL, T. K.; TURNER, D.; RUPP, R.; HOLLENBERG, S.; ZHUANG, Y.; LASSAR, A.:
The myoD gene family: Nodal point during specification of the muscle cell lineage. Science **251** (1991), 761-766.

DAVOLI, R.; FONTANESI, L.; ZAMBONELLI, P.; BIGI, D.; GELLIN, J.; YERLE, M.; MILC, J.; BRAGLIA, S.; CENCI, V.; CAGNAZZO, M.; RUSSO, V.:
Isolation of porcine expressed sequence tags for the construction of a first genomic transcript map of the skeletal muscle in pig. Animal Genetics **33** (2002), 3-18.

MERKS, J.W.M.:
One century of genetic changes in pigs and the future needs. In: The challenge of genetic change in animal production (W.G. Hill, S.C.; Bishop, B.; McGuirk, J.C.; McKay, G.; Simm, G; Webb, A.J.; Eds). Occasional publication no. 27. Britisch Society of Animal Science, Edinburgh (2000), pp. 8-19.

SELLIER, P.:
Genetics of meat and carcass traits. In: The Genetics of the Pig (Rothschild M.F.; Ruvinsky, A.; Eds), CABI publishing (1998), 463-510.

COUTINHO, L.; MORRIS. L. J.; MARKS, H. L.; BUHR, R. J.; IVARIE, R.:
Delayed somite formation in a quail line exhibiting myofiber hyperplasia is accompanied by delayed expression of myogenic regulatory factors and myosin heavy chain. Development **117** (1993), 563-569.

RIERA, L.; OBACH, M.; NAVASRRO-SABATÉ, A.; DURAN, J.; PERALES, J.C.; VIÑALS, F.; ROSA, J.L.; VENTURA, F.; BARTRONS, R.:
Regulation of ubiquitous 6-phosphofructo-2-kinase by the ubiquitin-proteasome proteolytic pathway during myogenic C2C12 cell differentiation. FEBS Letters **550** (2003), 23-29.

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