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gives new insights for muscle development

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Abstract
Bovine myogenesis can be characterized by five key stages during the foetal life. Thus, stages 60 and 110 days post conception (dpc) are witnesses of primary myoblasts and secondary myoblasts proliferation respectively. Stage 180 dpc shows the end of the proliferation step and is the point when the total number of muscle fibres is defined. The last third of bovine foetal life, encompassing 210 dpc and 260 dpc, is mainly characterised by a large metabolic and contractile differentiation step. If histological and biochemical experiments allowed the analysis of bovine myogenesis and allowed to display those stages, many questions remain about the proteomic mechanisms involved in the regulation of the:

i) proliferation of the two myoblasts generations,
ii) cellular transition proliferation/cellular alignment/fusion of the myoblasts in myotubes/differentiation,
iii) control of the total number of fibre.

A proteomic approach, based on the analysis of the semitendinosus muscle from Charolais cattle at key stages, was leading us to a classical two-dimensional gel electrophoresis and mass spectrometry strategy. We investigate the in vivo proteome of bovine muscle during semitendinosus muscle ontogenesis. Gene Ontology is a modern in silico tool that allows the attribution of a biological function to a single gene or a single protein but it can be used to characterize the main biological function of a particular cluster (composed of several proteins). Analysis of common expression profile of proteins and the use of gene ontology enables us to show some already confirm results and, above all, enables us to find other tracks to investigate the muscular development of large mammals in order to answer our issues.

Keywords: myogenesis, proteomics, proteome dynamics

Introduction
Muscle characteristics play a major role in meat quality. As bovine species present mature muscle at birth, most of muscle characteristics (including physiological and metabolic properties) of adult muscle are acquired during foetal life. Myogenic events
of bovine muscles have been described previously using mainly histological and immunological approaches. These studies drawn the conclusion that bovine myogenesis can be described according to the succession of key stages that reflect myogenic events observed for the majority of mammals. Thus, 60 and 110 days post conception (dpc) are characteristic of the proliferation of the primary and secondary myoblasts respectively. At 180 dpc, the total number of fibres is reached. At last 210 and 260 dpc are two main stages of i) contractile and metabolic muscle differentiation and ii) fibres maturation. In our study, these stages were kept in order to perform a proteomic investigation of global proteins modifications occurring during bovine myogenesis. Proteomic approaches are very powerful to give access to the final expression of a whole genome during complex process. Indeed, using two-dimensional gel electrophoresis (2DE) and large data exploration leading to the identification of 246 proteins by mass spectrometry, we described the proteome dynamics of foetal muscle during ontogenesis. The time course analysis of the 110 proteins common to the five key stages allowed the description of the main function and pathway involved in muscle formation thanks to the appropriate use of gene ontology and in silico tools.

Materials and methods
The proteomic investigation performed is described partly in CHAZE et al. (2008) in press. Briefly, four Charolais foetuses for each five stages were collected from cows slaughtered at the INRA experimental abattoir. Samples of the semitendinosus (ST) muscle were frozen in liquid nitrogen and stored at -80 °C, until protein extraction, done as previously described (BOULEY et al., 2004). Protein concentration was determined using the 2-DE Quant kit (Amersham, Uppsala, Sweden), an average protein concentration of 6.8±1.5 µg/µl was obtained from bovine foetal ST muscle. Proteins were separated by 2D gel electrophoresis. For the first dimension, iso-electrofocusing (IEF) steps were previously described by BOULEY et al. (2004) with the exception that after a desalting step (50 V, 7 h) proteins were separated under the following conditions, 200 V for 1 h, ramping to 500 V over 1 h, ramping to 1,000 V for 2 h, plateau at 1000 V for 1 h, ramping to 8,000 V over 9 h, plateau at 8,000 V until 73,500 V/h was reached. Second dimension SDS-PAGE was conducted on 12 % polyacrylamide gels in a Protean Plus Dodeca cell system (Bio-Rad, Hercules, USA) at 110 V and 15 mA per gel until the bromophenol blue migration front reached the bottom of all the gels. Two-DE gels were stained with G250 Colloidal Coomassie Blue. Four 2DE were performed for each foetus as technical replicates.

Gels were scanned at 300 dpi with an optical density calibrated ImageScanner (Amersham, Uppsala, Sweden). Image analysis was realised with ImageMaster 2-DE Platinum software (Amersham). Protein spots from the five stages were matched to a single reference gel. The reference gel was a physical gel conducted with an identical amount (in weight) of muscle protein, pooled from all samples analysed.

Data analyses of spots shared by the five stages were carried out using Principal Component Analysis and Hierarchical Clustering Analysis:
- In order to classify proteins presenting the same behaviour during bovine myogenesis, we performed a PCA analysis using the SPAD software.
- In order to group proteins presenting similar expression profiles across the five stages observed, hierarchical clustering analysis was used Hierarchical Clustering Analysis (HCA) was conducted using freely available PermutMatrix v.1.8.5 software (CARAUX et al., 2005).
HCA of the common protein spots was processed according to the Pearson distance. The Ward aggregation procedure was then used to construct the resulting dendrogram, as described previously (MEUNIER et al., 2007). The coomassie stained spots of interest were identified by MALDI-TOF mass spectrometry. Peptide mass fingerprint (PMF) of spots was determined using a Voyager DE Pro Matrix Assisted Laser Desorption Ionisation Time Of Flight mass spectrometer (Applied Biosystem). Protein identification was done automatically and validations of all proteins were done by hand. Bos taurus NR database was used to identify PMF of trypsin digested proteins. The Babelomics Fatigo+ tool was used to describe our data from clustering with the control vocabulary of Gene Ontology (AL-SHAHROUR et al., 2005). Groups of protein clusters exhibiting the same expression profile across the five stages of myogenesis were submitted to Fatigo+.

Results
This proteomic study of bovine myogenesis leaded to the detection of 496 protein spots and the identification by mass spectrometry of 246 proteins, mapped on the Figure 1. Among these, one hundred and ten proteins were shared by the five stages covering the whole bovine myogenesis.

Fig. 1: Cartography of the 246 proteins identified by mass spectrometry (MALDI-TOF) in semitendinosus muscle of bovines foetuses during muscle development.
Fig. 2: Principal Component Analysis of the 5 ages with 4 foetuses per stage

Fig. 3: Hierarchical Clustering Analysis of the 110 proteins common to the 5 stages
1, 2, 3, 4, 5 correspond to the different clusters grouping proteins with a same profile of expression across these stages. In green are proteins with low abundance, in red proteins with high abundance.
Analysis of these 110 proteins during muscle ontogenesis was performed using PCA and HCA analysis. In PCA, gels layout ensured a good gathering of 2D gel electrophoresis replicates since gels belonging to a specific stage were grouped together (Figure 2). Each stage was clearly separated in gels PCA experiments. Stage 60 and 110 dpc set at the opposite of stage 210 and 260 dpc while stage 180 dpc was set in the centre.

Data analysis using hierarchical clustering allowed the gathering of proteins exhibiting the same expression profile across myogenesis (Figure 3). In our study, the 110 proteins common to the five stages were classified in 5 distinct groups. The list of protein belonging to a particular cluster was submitted to the Babelomic’s suite in order to characterize each cluster by Gene Ontology controlled vocabulary. Thus, cluster 1 grouped proteins which abundance increased until 180 dpc and became stable during the last third of gestation. Functional analysis of this cluster indicated an increase of actin filament based process and also an increase of processes linked to cell death.

Cluster 2 consisted of proteins more and more abundant across myogenesis. Proteins grouped in cluster 2 were strongly associated to energy metabolism (cellular carbohydrate metabolic process, energy derivation by oxidation of organic compounds and coenzyme metabolic process). The main location of proteins forming this cluster was cytoplasm for the most part and mitochondria for some proteins.

Cluster 3 showed proteins which abundance increase until 180 dpc and decreased strongly at 260 dpc. GO terms characterising this cluster were linked to tissue organization (cytoskeleton organisation and biogenesis and regulation of striated muscle contraction). Cluster 4 exhibited proteins which abundance decreased across gestation. This cluster was particularly hard to be characterized, nevertheless this decrease in abundance was to be linked to developmental process (cell development) and mRNA splicing (mRNA processing and RNA splicing).

Cluster 5 displayed proteins which abundance was higher at 60 dpc and stable during the rest of myogenesis. This cluster could not be characterized using Gene Ontology vocabulary since it was composed of too little proteins (only 16 proteins) amongst whom five isoforms of albumin and two of transferrin were identified.

Discussion

This work represents the first detailed description of protein modifications during foetal myogenesis. It could constitute a reference not only for bovine but also for other species.

The PCA analysis described a good repartition of the different stages in accordance with previous data on bovine myogenesis. Stages 60 and 110 dpc are grouped and opposite to the other stages. The data of PICARD et al. (2002) through the analysis of different myosin heavy chain isoforms (MyHC) at cellular and tissue levels, demonstrated that at these stages the number of primary and secondary myotubes increased. This suggests, a proliferating and fusing activity of myoblasts at these stages which is coherent with the high expression of proteins involved in developmental process and mRNA processing and RNA splicing such as HNRH3 and Apolipoprotein B mRNA editing enzyme, at these stages. On the contrary, stages 210 and 260 dpc, grouped and opposite to 60-110 days, correspond to differentiation process as the abundance of proteins associated to energy metabolism increased during
this period. These are coherent with the previous data indicating an increase of oxidative and glycolytic enzyme activities during the last trimester of bovine gestation associated with contractile differentiation characterised by modifications of MyHC isoforms (GAGNIERE et al., 1997 and 1999).

These data confirm the key stages of 180 days which was previously identified as a stage of the determination of the total number of fibres in charolais foetuses by histochemical analysis (PICARD et al., 2002). We observe numerous modifications of protein abundances and changes in isoforms expression from this stage. Particularly, the expression of proteins involved in tissue organisation falls down from this stage. Interestingly, we observe changes in proteins involved in apoptosis until 180 days. This suggests that the total number of fibres is the result of a balance between proliferation and apoptosis of muscle cells as described in CHAZE et al., 2008 (in press). Among the proteins with an over abundance on 180 days pc, some could constitute good candidates as markers of the total number of fibres. For example, the proteins WARS, PARK7 or CLIC4 whose expression decreased after 180 days pc, could be good markers. Further analyses are in progress to confirm this hypothesis. These types of markers are potentially interesting as the measure of the total number of fibres by histochemical techniques is very complex. Scientists working on myogenesis are looking for this type of markers in order to analyse how to manage the number of fibres which is of crucial importance for meat production.

In conclusion, these proteomics data will be used in the comparison of others types of bovines such as cattle presenting a high muscle development. This will be of importance to explain the differences of muscle development in these different types of animals and to understand the origin of muscle hypertrophy. These data will constitute also, a good basis for studies in comparative biology of myogenesis in different species.

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