MATERNAL NUTRIENT RESTRICTION FOLLOWED BY REALIMENTATION FROM EARLY TO MID-GESTATION IN BEEF COWS IMPACTS PROTEOMES OF FETAL MUSCULAR AND ADIPOSE TISSUES

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Abstract – Perturbations of the prenatal environment may influence fetal muscle and adipose tissue (AT) development. This study investigated the effects of nutrient restriction and realimentation from early to mid-gestation on molecular events that underlie muscle and AT growth. On day 30 of pregnancy, 18 multiparous, non-lactating cows were fed at requirement (C; 100% NRC) or restricted (R; 60% NRC). On day 85, cows remained on control (CC; n = 5) or restricted (RR; n = 5) diets, or were realimented to control (RC; n = 5). A– proteomic analysis of the *Semitendinosus* and *Longissimus thoracis* muscles as well as perirenal and omental AT from 140 days-old fetuses was conducted using 2-DE and MS. The abundances of 28 and 34 proteins were modified (P<0.10) by maternal nutrition in muscles and AT, respectively. These proteins are involved in glucose or protein metabolisms, in the regulation of cell proliferation or apoptosis, and thus could be related to variations in the number and the size of myofibers and adipocytes. These results are of interest for the fetal programming of myogenesis and adipogenesis in general, and more specifically to the control of muscle and AT growth in meat producing animals.

Key Words – adipose tissue, muscle, growth fetal programming

I. INTRODUCTION

Producing meat animals with adequate muscular and adipose masses (i.e. lean-to-fat ratio) is an economic challenge for the beef industry. The lean-to-fat ratio is the result of a dynamic balance between the number and size of muscular and adipose cells. The number of muscle fibers is set during gestation [2], implying that any events that trigger a regulation or balance between the number of muscular and adipose cells must occur and be regulated during fetal life. The effects of manipulating maternal nutrition on the muscle and AT growth have been studied in sheep but rarely in cattle. Current knowledge shows that the most critical period for the impact of maternal nutrient restriction on muscle growth is before the half of pregnancy in ewes. It resulted in an increased diameter of fast myofibers, no change in diameter of slow myofibers and a decreased fast-to-slow myofiber ratio per unit area. Concomitantly maternal undernutrition, during early to mid-gestation (28 to 80 days) increased the fetal fat mass in near-term fetuses (for review see [3]). The molecular mechanisms associated with altered AT growth [4,5] and modified myofibers structures [1,6] begin to be revealed in ruminant fetuses. However the impact of nutrient restriction followed by realimentation on AT and muscle growths, as well as on their balance, remains to be unraveled [2]. Our objectives were to evaluate the effects of nutrient restriction during early gestation followed or not by realimentation to mid-gestation both on AT and muscle growth as well as on the related molecular events.

II. MATERIALS AND METHODS

Perirenal and omental AT, *Semitendinosus* (ST) and *Longissimus thoracis* (LT) muscles were sampled from bovine fetuses at 140 days post-conception (dpc) as previously described [1]. Briefly, Angus cows were randomly assigned to three dietary treatments at 30 days of pregnancy: cows maintained at 100% nitrogen and energy (NE) recommendations for maintenance and fetal growth from 30 to 140 dpc (CC, n= 5), cows

nutrient restricted to 60% NE recommendations until 85 dpc followed by realimentation to 100% NE recommendations up to 140 dpc (RC, n=5) and cows nutrient restricted to 60% NE from 30 to 140 dpc (RR, n= 5).

Proteomic experiments were assayed to identify molecular events modified by maternal nutrition that may affect AT and muscle growth, as well as a balance between their growths. Total protein were extracted as previously described [7], and were separated in a first-dimensional electrophoresis using 5-8 pH range. These proteins were separated in the second dimension on separating 12% polyacrylamide gels and were stained with G250 colloïdal coomassie blue. Protein spot detection and volume quantification were realized with SameSpots V4.5 software. We detected 368 muscular and 359 adipose well-resolved protein spots in the gel image. Trypsin-digested proteins were analyzed by online nanoflow liquid chromatography using an Ultimate 3000 RSLC (Dionex, Voisins Le Bretonneux, France) coupled to a LTQ VELOS mass spectrometer (ThermoFisher Scientific, Courtaboeuf, France). MS/MS ions were analyzed using Proteome Discoverer V1.4.1.14 and compared to *Bos taurus* nrNCBI database using MASCOT V 2.3. We have identified 98 adipose and 69 muscular proteins. Protein abundances were analyzed using variance analysis (ANOVA, R software) to compare diets whatever the anatomical site of AT and muscle. Proteins whose abundance changed with diet in CC *versus* RC or RR were considered significant at $P<0.10$. Data mining of proteomics results was performed using ProteINSIDE (www.proteinside.org) an online tool that we have developed to analyze lists of protein or gene identifiers from well-annotated species (human, rat, and mouse) and ruminants (cow, sheep, and goat) [8]. Briefly, ProteINSIDE gathers biological information stored in well-updated public databases for a systematic and integrative analysis of a protein's biological data, proceeds to annotations according to the Gene Ontology (GO) consortium to identify over expressed functions in a list of identifiers, predicts potentially secreted proteins that mediate signalization between cells or tissues, and searches for proteins interactions to identify proteins contributing to a process or to predict protein function.

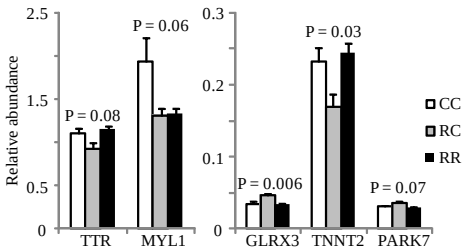
III. RESULTS AND DISCUSSION

At 140 dpc, absolute and relative (to body or eviscerated body weights) weights of perirenal and omental plus mesenteric AT were not modified by maternal nutrition. However an impact of maternal nutrition on muscle growth potential was sustained by a decreased fiber cross-sectional area of the *Infraspinatus* muscle in RC and RR fetuses compared to CC ones [1]. The abundance of 34 adipose (identified in omental or perirenal AT) and 28 muscular (identified in ST or LT muscles) proteins were modified by maternal nutrition (in RC or RR when compared to CC) and 4 (CCT6A, HBBF, PDIA3, RBP4) were retrieved both in AT and muscles. Thanks to the gathering of biological information from the main public databases, ProteINSIDE allows to identify at a glance proteins that could be key in the regulation of tissues growth. As an example for muscular proteins, structural proteins such as TNNT2 and MYL1 were highlighted, as well as proteins such as PARK7 that was proposed to be a regulator of the proliferation of the embryonic muscle cell lineage [9]. Isoforms of troponins, MYL1 and PARK7 were already identified in fetal bovine muscle [9], and the present study revealed that their abundances were regulated by maternal nutrition (Fig. 1).

The muscular proteins were annotated with enriched GO terms related to biological processes such as "muscle filament sliding" which encompassed 3 proteins (ACTC1, MYL1, TNNT2), "tricarboxylic acid cycle" (DLD, PDHB) and "glycolytic process" (ENO1, PGAM1), "negative regulation of apoptotic process" (ANXA5, PARK7, PSMA2), "cellular protein metabolic process" (PDIA3, VBP1 RPS12, CCT6A). In regards to these results, an increase in the abundance of glycolytic proteins was reported between 110 and 180 dpc to sustain myogenesis [9]. Moreover an increase in glycolytic and oxidative metabolisms was repeatedly reported to be associated with muscular hypertrophy in growing cattle [10]. Lastly an inhibition of apoptosis was proposed to regulate the number of muscular cells during fetal bovine myogenesis [9]. Present results show that abundances of proteins involved in these pathways are modified by maternal nutrition, and consequently could induce or be related to variations in the number and the

size of myofibers in bovine fetuses (see the abstract by B. Picard).

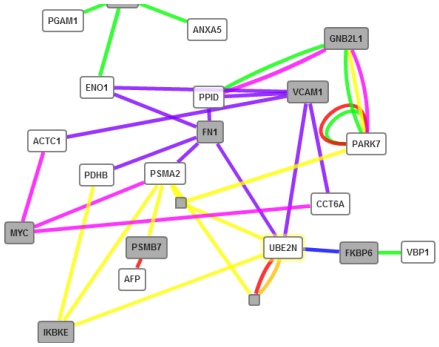
Figure 1. Abundance of TTR and GLRX3 in ~~adipose tissues~~AT and of MYL1, TNNT2 and PARK7 in muscles from fetuses



The adipose proteins were annotated with enriched GO terms related to biological processes "protein folding" and "cellular protein metabolic process" (PDIA3, CCT5, CCT3, CCT6A), "arginine catabolic process" (ARG1, DDAH2), "cell proliferation" (FSCN1, SERPINF1), "negative regulation of apoptotic process" (DDAH2, PSMB2). Pathways related to the metabolism of amino acids, as well as to rates of proliferation, apoptosis, and cell survival were already identified during the time course of bovine fetal adipogenesis [7]. However, even if pathways were similar, the majority of the proteins identified in the present study were not identified in our previous one. Among the newly identified proteins, GLRX3 known to regulate the function of the thioredoxin system, was never identified in AT. However, GLRX3 was proposed to protect cells against oxidative stress and to enhance cell cycle progression during late mitosis in mice embryos [11]. The thyroid hormone-binding protein, transthyretin (TTR) involved in the transport and availability of thyroid hormones to peripheral tissues was previously identified in fetal bovine AT [7] (probably as a blood protein) and was shown to have a modified abundance by maternal nutrition in the present study (Fig. 1). The thyroid hormones, thyroxine (T4) and triiodothyronine (T3), are essential for normal growth and development of the fetus [12,13]. Availability of T4 has been shown to regulate glucose oxidation in ~~adipose tissue~~AT of fetal pigs. Plasma T3 concentrations were positively related to a reduced efficiency of ATP production in AT of fetal sheep [12]. The variation in the abundance of TTR ~~in response to~~by maternal nutrition may modify the availability in thyroid hormones for fetal AT and consequently the metabolisms that sustain fetal adipogenesis.

To complement the results provided by the GO annotation, we looked for additional pathways and central proteins that could regulate myogenesis and adipogenesis, by analyzing the protein-protein interactions (PPI). We provide here results obtained for muscles (Fig.2).

Figure 2. Protein-protein interaction network that includes proteins identified in bovine fetal muscles (white boxes) and proteins external to our dataset (grey boxes). The network was filtered using high values of betweenness (1500, which quantifies how frequently a node is on the shortest path between every pair of nodes for detecting bottlenecks in a network) and closeness (0.1, which quantifies how short are the minimal paths from a given node to all others: a high closeness indicates that a node is close to the topological center of the network) centralities. Edges are experimental methods used to identify protein-protein interactions (one color by methods)



By querying databases IntAct, UniProt, and BioGrid (chosen for their high data curation) we identified 19 muscular proteins that were experimentally proven in Human to interact with 349 proteins that were outside of our dataset. The central (proteins that link the highest number of proteins or proteins that are involved in the shortness paths) proteins of this network were sorted (Fig. 2). The ~~central~~ role of PARK7 for myogenesis regulation is sustained by the present analysis, and could be related to the positive effect of Park7 on myofiber diameter and sarcomeric myosin expression reported *in vitro* [14] and the

Commenté [rw1]: Which subunit of the tetrameric TTR was detected?

Commenté [mubo2]: In fact TTR is an homotetramer so it is difficult to distinguish subunit by proteomic.

Commenté [rw5]: Please, explain why a central node is a key regulator of the myogenic process. ->DONE

Commenté [rw6]: Park7 was more expressed in RC compared to CC and RR; this suggests that Park7 could be regulated by refeeding of the mother. So would Park7 play a central role in case of nutritional changes only? What about the size of myofibers in RC and RR? ->DONE

Commenté [rw3]: What was the thyroid status of the fetuses? At day 140, does TTR actually carry thyroid hormones? Why should TTR, a plasma protein synthetized by the liver, actually be detected in these tissues? ->DONE

Commenté [mubo4]: The thyroid status was not assayed in the current experiment. However data reported in the reference 13 by Cassar-Malek and al, indicates that in bovine fetuses "plasma concentrations of reverse-triiodothyronine (rT3) and thyroxine (T4) increased during development from day 110 to day 210 or 260, respectively, whereas concentration of triiodothyronine (T3) increased from day 180 onwards

lower proportion of small fiber in RC group (see abstract by B. Picard). The ~~central~~ role of enzymes related to glycolysis (ENO1, PGAM1) and tricarboxylic acid cycle (LDHB) was also revealed by PPI network analysis. As new putative central proteins for myogenesis that were identified as nutritionally regulated in fetal muscles, we highlighted CCT6A, VBP1, UBE2N, that were never reported as muscular proteins and their role in myogenesis remains to be studied.
Lastly, PDIA3 in muscle, as well as PDIA3, TTR and SERPINF1 in AT, were identified as secreted proteins. Whether these proteins contribute to the regulation of myogenesis and adipogenesis or to the balance between these processes remains to be unraveled.

IV. CONCLUSION

This *in vivo* investigation of nutritionally regulated proteome during bovine myogenesis and adipogenesis provides new perspectives on the regulation of muscle and AT development. We have identified proteins with an abundance modified in response to maternal nutrition that could be key in the regulation of tissues growth, which remains to be proven by additional experiments.

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