# ANALYSIS OF GENE EXPRESSION IN PORCINE *M. SEMIMEMBRANOSUS* DIVERGENT IN INTRA-MUSCULAR FAT CONTENT

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**Abstract – Intra-muscular fat (IMF) content is significantly correlated with aspects of pork palatability, such as flavour, juiciness and overall acceptability. The objective of this study was to compare gene expression profiles of *M. semimembranosus* (SM) muscle of animals divergent for IMF, with a view to elucidating the transcriptomic mechanisms regulating this trait in porcine muscle. The animal model was derived through the imposition of a low protein (LP) diet during the grower-finisher stage in Duroc gilts (n=5), in comparison to a high protein diet (HP) (n=6). SM tissue was preserved at slaughter, RNA was extracted, processed and hybridised to Affymetrix porcine GeneChip® arrays, followed by statistical analysis using *Bioconductor* in *R*. The *puma* method was used to estimate fold changes and *P*-like values for differentially expressed transcripts and probesets were annotated using *Anexdb*. Transcript differences were validated for a subset of genes by qPCR. The IMF content of SM muscle was significantly increased in LP, compared to HP animals ($P < 0.001$). Microarray results indicated that a total of 542 annotated genes (191 up- and 351 down-regulated) were significantly altered in expression between conditions. Overall, numerous signalling pathways were modulated in relation to IMF content. The arachidonic acid pathway, important for growth and development, was largely down-regulated in high IMF muscle. As expected, genes in the leptin signalling pathway, the PPARα/RXRα activation pathway and stearoyl coA desaturase were up-regulated in high IMF tissue. Identified panels of genes have potential as biomarkers of fat deposition in muscle.**

**Key Words – lysine, marbling, transcriptomics**

## I. INTRODUCTION

In the last 30 years, intensive selection for lean growth has dramatically altered many porcine muscle characteristics, including a reduction in the levels of intramuscular fat to the extent that it is now commonly observed that fat content in commercially important cuts of pork is less than 1.5% in many breeds and lines [1], a level that has been proposed as a threshold below which palatability is not acceptable [2]. Identification of the transcriptomic response in muscle divergent in IMF would provide insight on the genes influencing this complex trait. Even in modern commercial breeds, muscle fat content may be altered by alterations in animal physiology in response to external factors such as nutritional challenge. Dietary protein restriction, relative to overall energy content in the diet, is an approach which has been show to result in an increase in IMF content in porcine muscle [3]. The Duroc breed is characterised by moderate levels of IMF compared to other commercial breeds [4, 5] and is therefore a useful breed for exploration of muscle lipid metabolism in pigs. The aim of this study was to explore the muscle transcriptomic response in porcine *M. semimembranosus* with divergent IMF content, in order to gain insight into the molecular mechanisms underpinning IMF content.

## II. MATERIALS AND METHODS

*Dietary groups and IMF assessment*

Eleven purebred Duroc gilts were assigned to one of two dietary treatments on the basis of initial body weight (BW) (46.3 ± 4.2 kg) for 63

days. High (HP) and low protein (LP) diets contained 13.0 and 7 g/kg of total lysine. All the pigs were removed for slaughter on day 63 of the experiment. Animals were transported to the pilot-scale abattoir at Teagasc Food Research Centre, Ashtown. M. *semimembranosus* (SM) tissue was removed from each carcass and chopped under RNAse free conditions, preserved in RNALater® (Ambion) within 10 min post-slaughter, placed on ice, kept overnight at 4 °C, and then stored at -20 °C until RNA extraction. Muscle intramuscular fat and moisture concentrations were determined using the Smart System 5 and NMR Smart Trac rapid fat analyser (CEM Corporation USA). Protein concentration was determined using a LECO FP328 (LECO Corp., MI, USA).

*Gene expression*

High quality RNA (RIN 8.0) was extracted from SM tissue, processed and hybridised to the Affymetrix GeneChip® porcine array. PUMA was used to estimate fold changes and p-like values [6]. Differentially expressed transcripts were annotated using ANEXdb [7]. Ingenuity® Pathway Analysis™ (www.ingenuity.com), DAVID [8] and MetaCore™ (GeneGo Inc.) were applied for ontology analysis. Ten selected differentially expressed genes and three non-changing genes were analysed by QPCR with Power SYBR® Green on the AB7500 instrument.

## III. RESULTS AND DISCUSSION

*IMF and diet*

Phenotypes were analysed using the General Linear Model procedure of SAS. The two treatment groups were balanced for body weight before the dietary trial, and there was no significant effect of diet on carcass weight at slaughter ($P > 0.05$). The LP and HP diets did, however, have a significant effect on the composition of SM muscle. SM muscle derived from animals on the LP diet had significantly higher levels of IMF in comparison to muscle from the HP diet (3.60 ± 0.38 % versus 1.92 ± 0.35 %, $P < 0.001$). There was no effect of dietary treatment on protein or moisture content in the muscle. This indicates likely variation in the modulation of biological pathways relevant to protein and fat metabolism influenced by breed, diet and muscle type, suggesting that there is considerable scope for exploration of the biochemical basis of intramuscular fat deposition by gene expression analysis of these samples.

*Differential gene expression*

744 transcripts (542 annotated by ANEXdb) were differentially expressed (DE) in high and low IMF muscle with fold changes ranging from -6.29 to 5.14 (130 genes altered more than 1.5 fold). The three most noticeably up-regulated genes in high IMF muscle were ankyrin repeat and sterile alpha motif domain containing 1B (*ANKS1B*) with a 5.14 fold change, aurora kinase A interacting protein 1 (*AURKAIP1*) gene with fold change of 4.58 and suppressor of cytokine signalling 3 (*SOCS3*) gene with a 4.85 higher abundance in high IMF animals. The most dramatically down-regulated gene identified on the microarray in higher IMF tissue was stathmin-like 2 (STMN2) with a -6.291 fold change. STMN2 has been suggested play a role in the regulation of the adipocyte/osteoblast balance. The second most repressed gene was potassium voltage-gated channel, Shal-related subfamily, member 2 (*KCND2*) with a -3.254 fold change, which was followed by solute carrier family 19, member 3 (*SLC19A3*) gene with a -2.971 fold change.

Real-time PCR fold changes for 13 selected genes displayed similar magnitude and direction compared to the microarray data (Table 1).

Table 1: Realtime PCR validation of selected genes
*, p<0.05; **, p<0.01; ***, P<0.001

| | Fold change | | P-value | |
|---|---|---|---|---|
| Gene | Array | qPCR | Array | qPCR |
| MPHOSPH6 | 1.21 | 1.41 | *** | ** |
| IQGAP2 | 1.42 | 1.36 | *** | 0.09 |
| UBE2CBP | 1.18 | 1.29 | * | ** |
| CBX5 | 1.36 | 1.21 | *** | 0.09 |
| SATB2 | 1.31 | 1.30 | *** | 0.10 |
| SLMAP | 1.12 | 1.32 | NS | NS |
| Adiponutrin1 | 3.1 | 1.91 | * | * |
| ANGPTL4 | -3.64 | -2.36 | * | ** |
| SCD | 2.9 | 6.19 | * | 0.07 |
| PRCOR-like | -2.3 | -2.46 | *** | * |
| BTG2 | 1.75 | 2.42 | * | * |

Almost twice as many annotated transcripts (351) were downregulated in high IMF muscle, compared to up-regulated (191). This suggests that a restriction of protein in the diet causes a relatively greater repression of normal muscular pathways compared to a stimulation of specific transcriptomic responses.

*Molecular and cellular functions of differentially expressed genes*

The top functional categories for up-regulated genes related to 'molecular transport' (20 genes), 'DNA replication' (20 genes) and 'cell death' (43 genes). 'Carbohydrate metabolism' was also significant (18 genes). Several of the functional categories down-regulated in high IMF tissue relate to the restriction of protein which limits protein synthesis e.g. 'cell cycle' (36 genes), 'DNA replication' (17 genes) and 'cellular assembly and organisation' (30 genes). 'Lipid metabolism', was represented in both up- and down- regulated gene lists with upregulated genes (8 genes) including SCD and LEP, while down-regulated genes (10 genes) were particularly relevant to unsaturated fatty acid metabolism.

*Canonical pathways associated with IMF content*

Diverse signalling pathways were significantly represented among the differentially expressed genes.

Table 2: Significant canonical signalling pathways in overall DE genelist

| Ingenuity® canonical signalling pathway | *P*-value | % DE |
|---|---|---|
| Factors Promoting Cardiogenesis | 0.00206 | 8.51 |
| Antiproliferative Role of TOB in T Cell Signalling | 0.00468 | 15.4 |
| Alanine and Aspartate Metabolism | 0.00608 | 5.68 |
| HIF1α Signalling | 0.00664 | 7.27 |
| Nicotinate and Nicotinamide Metabolism | 0.0175 | 5.15 |
| Apoptosis Signalling | 0.0247 | 6.67 |
| PPARα/RXRα Activation | 0.0308 | 5 |
| Arachidonic Acid Metabolism | 0.0365 | 3.57 |
| Nitrogen Metabolism | 0.0386 | 3.01 |
| Prolactin Signalling | 0.0388 | 6.49 |
| p38 MAPK Signalling | 0.0433 | 6.25 |
| ATM Signalling | 0.0467 | 7.55 |
| NF-κB Activation by Viruses | 0.0475 | 6.00 |
| Intrinsic Prothrombin Activation | 0.0488 | 8.60 |
| HGF Signalling | 0.049 | 5.71 |

Several of these pathways relate to growth (Table 2). Genes in the PPARα/RXRα activation pathway were up-regulated in high IMF tissue (Table 2). The cellular response to hypoxic stress was also up-regulated (e.g. HIF1α, apoptosis signalling).

In contrast, only four metabolic pathways were significantly represented in up- and down-regulated genelists analysed separately (data not shown). Arachidonic acid signalling is important for cellular growth and differentiation and was generally down-regulated (e.g. ALOX12, GPX3, PLOD3 genes). Nicotinamate metabolism was also significant for down-regulated genes, suggesting a general repression of anabolic processes. Amino acid metabolism was significant for up-regulated genes, indicating a transcriptomic response to the experimental limitation of dietary amino acids.

*Top networks associated with IMF*

Analysis of molecular networks was performed using IPA. All 542 genes DE at the 0.05 level were analysed using IPA software to identify networks of differentially expressed genes. Twenty-five significant networks were identified with 18 having 15 or more of the DE genes in the network.

Table 3: DE genes in the most significant network

Genes in bold are up-regulated; genes in plain text are down-regulated; genes in grey are network members not observed differentially expressed in the experiment

| Network 1: Molecular Transport, Lipid Metabolism, Small Molecule Biochemistry |
|---|
| AKR1C1, ATP1B1, ATPase, CBX2, **CBX5**, **CELF2**, COL3A1, COL5A3, Collagen type I, CSDC2, CYP2C19, DAZAP2 (includes EG:9802), DNAJB6, **DSN1**, **ELP2**, ETFDH, Histone h3, Histone h4, HOXA9, Hsp70, **KCNC2**, **LEP**, Mediator, **NR3C2**, NSL1, Pka, RBPMS, RNA polymerase II, RSF1, Rxr, **SERINC2**, SMC4, SPAST, TARBP1 (includes EG:6894), TMSB10 |

The genes DE in the most significant network are shown in table form in Table 3 and network relationships illustrated in Figure 1. Leptin plays key roles in fatty acid homeostasis and is a hub molecule in this network. Indeed, several studies

have shown that high circulating levels of leptin inhibit fatty acid esterification in rodent and porcine skeletal muscle and promote lipid oxidation. Here leptin shows potential as a marker of high IMF tissue.

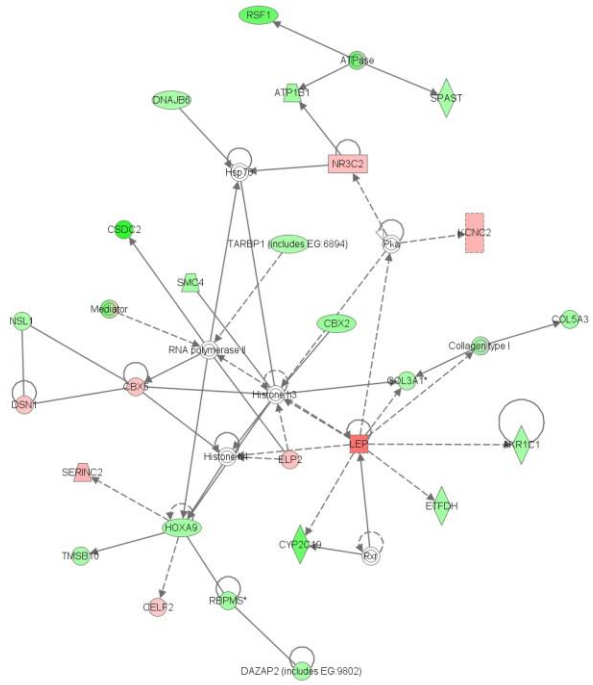


Figure 1: DE genes in the most significant IPA network. Genes in red are up-regulated in high IMF muscle; genes in green are down-regulated in high IMF muscle; genes in grey are network members not observed in the experiment.

## IV. CONCLUSION

These data confirm that restriction of dietary protein results in an accumulation of IMF content. This process is associated with a stimulation of expression of genes with functions in molecular transport, lipid metabolism, carbohydrate uptake and storage and a suppression of cellular growth-related processes. The expansion of adipose tissue in high IMF muscle may lead to an increase in leptin signaling, the oxidative stress response and apoptosis pathways.

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