Protein restriction differentially modifies liver transcriptome at different stages of the growing period of Duroc x Iberian crossbred pigs

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Summary

Protein restriction during the growing period reduces feeding costs even diminishing the growth rate without a detriment to intramuscular fat content, which is a key meat quality trait. The effect of a low protein diet compared with a standard one in the liver transcriptome of Duroc x Iberian crossbred pigs was studied in order to identify gene pathways and networks modified by protein restriction at two different stages of the growing period. A total of 219 and 611 DE genes and potentially new isoforms were identified in RNA samples of liver proceeding from two batches of pigs slaughtered at 45 and 90 kg of body weight, respectively. The GO enrichment analyses and Genome-Scale Metabolic Network (Recon) revealed an enrichment of DE genes in pathways related with the immune system and lipogenesis at 45 kg, and with growth and with cholesterol metabolism at 90 kg. These results point out that the effect of a protein restriction on liver transcriptome is conditional to the growth stage. It should be taken into account for identifying candidate genes related to pig growth and fatness.

Keywords: Iberian pigs, protein restriction, transcriptome

Introduction

Iberian pigs have low growth rates and substantial fat depots which are associated with excellent sensorial qualities of meat and dry-cured meat products (Lopez-Bote, 1998). The dominant trend in the Iberian porcine sector has been focused to raise the production of Duroc x Iberian crossbred pigs in order to get a balance between good growth and carcass performance along with desirable meat and fat quality.

The Standard Quality rule of Iberian pig in Spain establishes a minimum age and body weight (BW) at slaughter of 10 months and 115 kg, respectively. However, crossbred animals fed *ad libitum* can achieve this weight at 8 months making it very difficult to strictly comply with this regulation. A possible solution is to apply protein restriction during the growing period to decrease the growth of the animal (Lebret, 2008). The protein restriction has been associated with an increase in the intramuscular fat content which is a very important meat quality trait (Madeira *et al.*, 2013).

Identification of gene pathways and networks whose expression is modified due to an alteration in the diet would provide a better understanding of the physiological and biochemical processes involved in growth and fat deposition. In the current study we analyse

the effect of a low protein diet in the transcriptome of liver samples and the networks of differentially expressed genes in pigs at 45 and 90 kg of BW.

Material and methods

Animal material, diets, RNA isolation and sequencing

Twenty crossbred pigs (Duroc x Iberian) with initial body weight of 25 kg were assigned to two different dietary treatments during the growing period: 1) a control diet (C) based on wheat, barley and soy with a formulation following FEDNA recommendations (3.180 kcal/kg EM, 16% protein content (PC) and 0.82% Lysine) and 2) a low protein diet (LP) with the same energy than the control one and lower levels of PC (11%) and lysine (0.60 %). The animals had *ad libitum* access to feed and water. There were two slaughter batches according to BW, one at 45 kg (middle of the growing period) and the second one at 90 kg (end of the growing period). Pigs slaughtered at 45 kg from both diets had an average age of 103 (±0.01) days, while those slaughtered at 90 kg had average ages of 165 (±0.08) and 174 (±0.08) for C and BP diets, respectively. A total of 10 animals, five per diet treatment, were slaughtered at each BW class.

Liver samples were collected at slaughter and stored at -80ºC. Total RNA was extracted using Ribopure High Quality total RNA kit (Ambion, Austin, TX). The integrity of the RNA was assessed with an Agilent 2100 Bioanalyzer device (Agilent Technologies, Santa Clara, CA). Paired-end libraries were prepared to be sequenced on an Illumina Hi-Seq 2000 (Fasteris, Plan-les-Ouates, Switzerland) with five samples per lane generating paired-end reads of 75 bp.

The quality of the raw sequencing data was assessed with *FastQC* and trimmed using *Trim Galore*. Filtered reads were mapped against the pig reference genome (Sscrofa11.1 using *TopHat* v2.1.0 (Trapnell *et al.*, 2009) through the alignment of the reads first to the ENSEMBL (11.1) transcriptome annotation. Transcripts were assembled using *Cufflinks* v2.2.1 (Trapnell *et al.*, 2012). *Cuffcompare* tool was used to classify the transcripts.

Differential Expression Analysis, Gene Functional Classification and Network Analyses

The *Cuffdiff* tool was used to calculate expression values and perform the differential expression analyses of the annotated genes and the newly predicted isoforms between the C and LP diets at 45 and 90 kg. The annotated genes and new isoforms with a minimum mean group expression of 0.5 FPKM and a fold change of the expression differences between C and LP of 1.2 were filtered. The R package *q*-value (Storey and Tibshirani, 2003) was employed for adjusting the significance to multiple testing. Those genes and new isoforms with a nominal *p*-value lower than 0.05 and a *q*-value lower than 0.10 were considered as differentially expressed (DE).

Functional analyses of the DE genes were carried out by examining GO enrichment with FatiGO using GO database. In addition, STRING was used to generate networks and explore the relationships between the proteins codified by the DE genes.

Results and Discussion

Characterization of liver transcriptome and differential expression analyses

A total of 828.93 and 898.15 million reads were obtained after trimming and filtering in the liver samples at 45 and 90 kg, respectively. Approximately 94.13 % of reads were mapped against the pig reference genome (build 11.1) at both BW. More transcripts were identified at 45 kg (184,792) than 90 kg (152,335). Table 1 shows the classification made by *Cuffcompare*. The percentage for category observed at each BW was very similar. A high percentage of transcripts corresponding to potentially new isoforms, transcripts falling within an intron and within intergenic regions (72.90% and 69.62%) was observed. These large numbers should be explained by an incomplete annotation of the available porcine genome annotation and they were also observed in other studies both in liver and other tissues (Ramayo-Caldas *et al.*, 2012; Corominas *et al.*, 2013; Pérez-Montarelo *et al.*, 2014). The differential expression analyses of the annotated genes shown a total of 152 DE genes in the liver transcriptome of pigs slaughtered at 45 kg BW, while 78 of these genes were down-regulated in the LP group, 74 were upregulated in these animals. On the other hand, a higher number of DE annotated genes were observed at 90 kg BW, 112 of 230 DE genes were downregulated in LP group and 118 upregulated in LP group. In addition to this, 67 and 381 DE potentially new isoforms were detected at 45 and 90 kg, respectively. Taking into account both growth stages, there were just two DE genes and new isoforms shared among the pigs slaughtered at 45 and 90 kg (Figure 1).

Biological interpretation

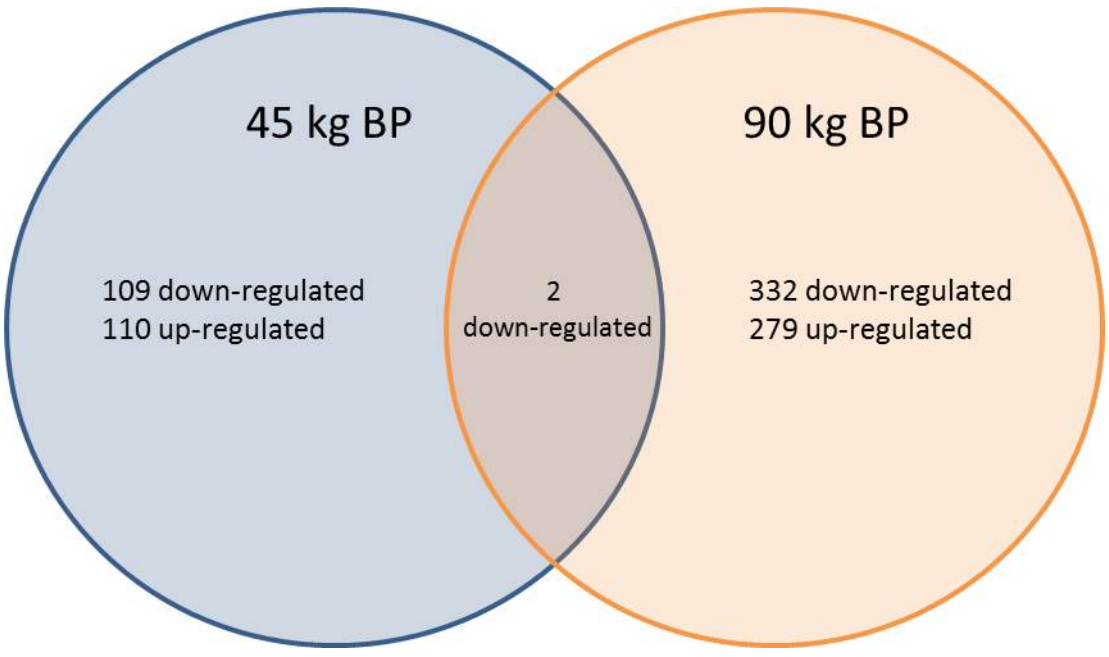
The GO enrichment analyses revealed different biological pathways at 45 and 90 kg. While at 45 kg there are a large number of biological processes enriched in DE genes related with immune system as defense response to virus (GO: 0051607) or response to type I interferon (GO: 0034340), at 90 kg the many GO biological terms are related with cholesterol metabolism and growth processes as sterol metabolic process (GO: 0016125), cholesterol metabolic process (GO: 0008203), skeletal muscle tissue development (GO: 0007519) and skeletal muscle organ development (GO: 0060538). In addition, the Genome-Scale Metabolic Network (Recon) revealed an enrichment of genes upregulated in the LP group involved in the *fatty acid elongation* pathway at 45 kg and in the *cholesterol metabolism* pathway at 90 kg. This indicates that protein restriction activates liver lipogenesis at the beginning of the growing period changing to fatty acids storage as cholesterol at the end of this period. When we analyze the interaction between the proteins codified by DE genes at the two BW, different networks are shaped, two main networks were detected at 45 kg, one was composed by MX1, OAS2, RSAD2, DDX58, USP18 and IFIT1 proteins, which are involved in immune system and the other one composed by SCD, FASN, ACSL1, THRSP, GPAM and DIO2 proteins, involved in fatty acid synthesis. On the other hand, a network composed by RUSC1, HMGCR, HMGCS1, DHCR24, SQLE, CP7A1, SOAT2 and CYP20A1 proteins related with cholesterol and sterol metabolic processes were observed at 90 kg.

Many studies on nutrigenomics or search of candidate genes are based on transcriptome analyses at the end of the animal lifespan. According to our results on liver transcriptome, these studies should be sequentially carried out at different growth stages since the transcriptome patterns may be changing with the age. The ensemble of results of sequential analyses allows identify a larger number of candidate genes for the traits of interest.

Table 1. Classification of the transcripts identified in the liver samples at two different body weights in relation to the Ensemble annotated pig genes. <Times 12, tables should be ended by a period>

	45 kg		90 kg	
	Transcripts, n	Transcripts %	Transcripts, n	Transcripts %
Complete match of intron	12,626	6.83	12,502	8.21
Contained in the reference	8,369	4.53	8,828	5.80
Potentially novel isoform	69,439	37.58	58,936	38.69
Pre-mRNA	6,482	3.51	5,169	3.39
Exon falling into an intron	48,102	26.03	32,724	21.48
Generic overlap	2,614	1.41	2,446	1.61
Polymerase fragment	2,275	1.23	2,216	1.45
Intergenic fragment	17,177	9.30	14,401	9.45
Exonic overlap with reference on opposite strand	2,508	1.36	2,160	1.42
Multiple classifications	15,200	8.23	12,953	8.50
Total	184,792	100.00	152,335	100.00

Figure 1. Genes and new isoforms down and upregulated in pig liver transcriptome under a low protein diet at 45 and 90 kg of BW and common to both weights.



List of References

Corominas J., Ramayo-Caldas Y., Puig-Oliveras A., Estellé J., Castelló A., Alves E., Pena R. N., Ballester M. and Folch J. M. (2013) Analysis of porcine adipose tissue

transcriptome reveals differences in de novo fatty acid synthesis in pigs with divergent muscle fatty acid composition. *BMC Genomics,* 14**,**(1): 843.

Kampman-van de Hoek E., Sakkas P., Gerrits W. J., van den Borne J. J., van der Peet-Schwering C. M. and Jansman A. J. (2015) Induced lung inflammation and dietary protein supply affect nitrogen retention and amino acid metabolism in growing pigs. *Br. J. Nutr.,* 113**,**(3): 414-425.

Lebret B. (2008) Effects of feeding and rearing systems on growth, carcass composition and meat quality in pigs. *Animal,* 2**,**(10): 1548-1558.

Lopez-Bote C. (1998) Sustained utilization of the Iberian pig breed. *Meat Sci.,* 49: S17-S27.

Madeira M. S., Pires V. M., Alfaia C. M., Costa A. S., Luxton R., Doran O., Bessa R. J. and Prates J. A. (2013) Differential effects of reduced protein diets on fatty acid composition and gene expression in muscle and subcutaneous adipose tissue of Alentejana purebred and Large White× Landrace× Pietrain crossbred pigs. *Br. J. Nutr.,* 110**,**(2): 216-229.

Pérez-Montarelo D., Madsen O., Alves E., Rodríguez M. C., Folch J. M., Noguera J. L., Groenen M. A. and Fernández A. I. (2014) Identification of genes regulating growth and fatness traits in pig through hypothalamic transcriptome analysis. *Physiol. Genomics,* 46**,**(6): 195-206.

Ramayo-Caldas Y., Mach N., Esteve-Codina A., Corominas J., Castelló A., Ballester M., Estellé J., Ibáñez-Escriche N., Fernández A. I. and Pérez-Enciso M. (2012) Liver transcriptome profile in pigs with extreme phenotypes of intramuscular fatty acid composition. *BMC Genomics,* 13**,**(1): 547.

Storey J. D. and Tibshirani R. (2003) Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences,* 100**,**(16): 9440-9445.

Trapnell C., Pachter L. and Salzberg S. L. (2009) TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics,* 25**,**(9): 1105-1111.

Trapnell C., Roberts A., Goff L., Pertea G., Kim D., Kelley D. R., Pimentel H., Salzberg S. L., Rinn J. L. and Pachter L. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.,* 7**,**(3): 562-578.