GENE NETWORK ANALYSIS FOR MARBLING DEVELOPMENT USING GENE EXPRESSION (RNA-SEQ) IN HANWOO

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SUMMARY

This study aimed to use co-expression networks to reach a better understanding of the genetic mechanisms underlying marbling development and assess the effect of different diets on the transcriptomic profile and the resulting phenotype. We evaluated development of marbling in *Longissimus dorsi* muscle extracted from 45 Hanwoo steers at 8, 12, 18, 24 and 30 months of age. The effect of two different feeding conditions (high/low feeding) were evaluated on the gene expression in the relation with the marbling score. In the four groups according to the marbling score (MS) and the feeding treatment: HighFeed_HighMS, HighFeed_LowMS, LowFeed_HighMS, and LowFeed_LowMS we found 818, 928, 899, and 946 co-expressed genes respectively. The activity of modules changed significantly with the age indicating different expression profiles for genes involved in muscle growth (*PI3K-Akt signaling*, *Focal adhesion*, *ECM-receptor interaction*), metabolic regulation (*Biosynthesis of amino acids* and *Glutathione metabolism*) and lipid deposition (*Fatty acid metabolism*, *Regulation of lipolysis in adipocytes*) through the animal development. The effect of the feeding condition in animals that developed low MS showed the activation of pathways related to stress and maintenance of cell homeostasis under nutritional limitations, while pathways involved in fat deposition and lipid mobilization where observed under high feeding.

INTRODUCTION

In beef cattle, the evaluation of intramuscular fat, measured as marbling score, is an important indicator of meat quality and achieving higher levels of this trait is an economical incentive for producers. Improving the knowledge of the genes and pathways involved in the development of economically important traits can potentially help to improve management strategies, genomic selection, or molecular tools to improve beef production. There have been some studies that attempted to describe the mRNA (Lim *et al.* 2015) and miRNA (Seong *et al.* 2016) abundances in muscle samples from high and low phenotypes. However, all these studies were performed using tissue from animals at age of slaughter, showing the final phenotype (high or low marbling). The use of gene expression (RNA-seq) could be applied in the identification of markers for the onset of marbling at younger ages. Since differences in marbling are subtle at early ages, the analysis of co-expressed genes with the identification of networks could be more informative to explain the development of marbling and find markers than the analysis of differentially expressed genes alone. Differential expression analysis relied on big changes in expression between conditions, while in the co-expression analysis it is possible to identify the genes with similar expression profile which are more likely to be involved in the same metabolic pathway, have related function, may have been co-regulated and identifying them can assist in the finding of hubs or molecular targets (Russo *et al.* 2018).

MATERIALS AND METHODS

RNA sequences were obtained from previous study (Lim *et al.* in print). Briefly, 45 Hanwoo steers were grown on high (23 steers) and low (22 steers) energy diets from eight months of age until slaughter at 30 months. Muscle samples were taken from *Longissimus dorsi* at 8, 12, 18, and 24 by biopsy of the tissue, while muscle was sampled after slaughter at 30 months. The samples were sequenced in Illumina HiSeq200 to obtain paired-end reads of 100 bases pairs. Standard procedures were followed on the reads for quality control, cleaning, mapping and assembly (Lim *et al.* in print). Analysis was done for groups differentiated by diet (High vs Low Feed) and marble score at slaughter (High vs Low MS). The co-expression networks were performed separately for each sample group (HighFeed_HighMS, HighFeed_LowMS, LowFeed_HighMS and LowFeed_LowMS). For each group, we normalized the gene expression counts and filtered out the genes with low expression as well as the genes with low variance resulting in around 13,000 expressed genes. Pearson correlations were calculated on the logarithmic copies per million (lcpm) and the pairs of genes with a correlation ≥ 0.8 were selected for subsequent analysis. Similarities between these gene expression was used to identify modules of genes with similar expression profile by a dynamic tree cut. The biological role of the selected genes in each module was found through an over representation analysis to identify the pathways involved (adjusted P-value <0.05). The activity of the genes in each module, its activation or repression according to the age, was evaluated with a gene set enrichment analysis (GSE) using the R Package CEMItool (Russo *et al.* 2018). This analysis ranked the genes according to the correlation of their expression with the phenotypic class (high or low marbling) and determined whether the genes of each module tend to be at the top or bottom of the ranked list. The normalized enrichment score (NES) will be higher as the gene in the module is found in the ranked list; alternatively, the score value is negative if the genes are not found in the list. The score value could be zero if the set of genes are randomly distributed in the list. Finally, network graphs were made from the co-expression information and combined with information of protein-protein interaction extracted from the STRING v11.0 database to identify the hub genes (representing genes with interactions with multiple other genes).

RESULTS AND DISCUSSION

We found a similar number of genes selected for each of the four groups after filtering out low expression and variance, and keeping the genes that are highly correlated (Table 1). In general, most of the genes belong to module one, and there were "not-correlated" genes (NC) in each group. To assess the activity of the genes in each module and across ages we performed a gene set enrichment analysis (GSEA) for each group. The GSEA results showed variation in the normalized enrichment score indicating that the activity of the genes changed according to the age of the animals (Figure 1) suggesting that their expression have an effect in the growth of muscle and marbling. Every group presented modules with high activity at 30 months of age indicating also association with the final marbling phenotype. Genes with potential role in marbling development because of their activity at 30 months (red color) could be found in the modules M2, M3, and M5 for HighFeed_HighMS group; M3 in HighFeed_LowMS group; M1 and M3 in LowFeed_HighMS; while M2, M3, and M5 were identified in LowFeed_LowMS group.

Table 1. Number of genes and pathways represented for each sample group

Group	Genes	Pathways	M1	M2	M3	M4	M5	M6	NC
HighFeed_HighMS	818	47	172	160	152	122	101	49	62
HighFeed_LowMS	928	62	595	131	63	62	38	0	39
LowFeed_HighMS	899	78	495	150	69	61	0	0	124
LowFeed_LowMS	946	55	395	245	167	55	43	0	41

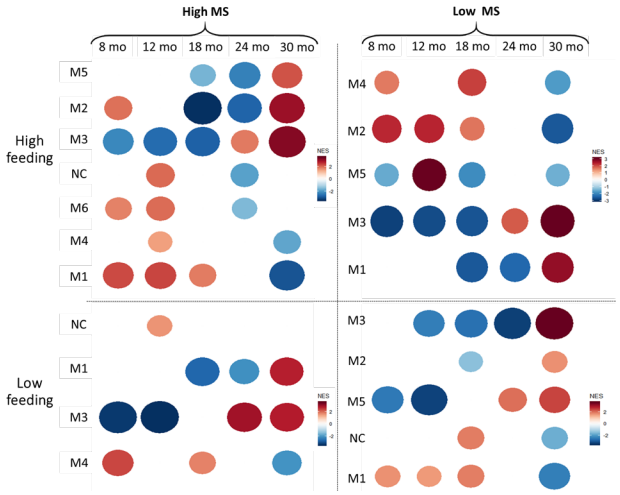

Figure 1. Gene set enrichment analysis showing the modules (M) and the normalized enrichment score (NES); NC= no correlated

We performed an over-representation analysis to determine which processes are mostly associated with de development of marbling in each group and we investigated if these pathways are affected by the feeding condition. To compare the pathways between groups, we selected 40 pathways with an important role in the muscle growth and the development of marbling (Figure 2).

We observed the presence of genes in multiple pathways showing that there is a cross-talk/interaction between them. Nine pathways reflected the conserved process involved in the morphology and growth of the skeletal muscle in all the groups: *PI3K-Akt signaling*, *Focal adhesion*, *ECM-receptor interaction*, *Carbon metabolism*, *Biosynthesis of amino acids*, *Glutathione metabolism*, *Fatty acid metabolism*, *Regulation of lipolysis in adipocytes*, and *Pentose phosphate*. Interestingly, there were also pathways represented exclusively in each group (except for HighFeed_LowMS). In the HighFeed_HighMS group the physiologic response under to the HighFeed diet activated important pathway involved in transport of glucose, body weight, fat deposition, and vasculature: *Apelin signaling*, *Rap1 signaling*, *FoxO signaling*, *Relaxin signaling*, and *Insulin resistance*. In skeletal muscle the entry of glucose is promoted by apelin which also affect the activity of FOXO1 gene (Hwangbo *et al.* 2017). The gene Rap1 have been reported in the control of body weight and metabolic regulation in mice (Yeung *et al.* 2013). The genes involved in pathways related to stress and low energy disposition (i.e. *HIF-1 signaling*, *Protein processing in endoplasmic reticulum*, *Alanine, aspartate and glutamate metabolism*) were identified in steers that developed low MS under low feeding conditions. Special attention was focused on the LowFeed_HighMS group since these represent animals with a high marbling score even under under low feeding conditions. In this group, the pathways *Starch and sucrose metabolism*, *Adipocytokine signaling*, *Pantothenate and CoA biosynthesis*, and *Oxidative phosphorylation* seems to have an important role in the regulation energy metabolism and deposition of intramuscular fat. The hub gene ADIPOR2 seems to have an important function in the *Adipocytokine signaling* (Figure 2). ADIPOR2 is an adipocytokine which expression affects lipid accumulation, the activity of PPAR-α signaling pathway (Ouchi *et al.* 2012; Cao 2014). Also in this pathway, the gene PGC-1α is a key regulator of the conversion of muscle fiber types from glycolysis (uses glycolysis as energy source) into oxidised (use fatty acid oxidation to produce energy) muscle fibers (Gu *et al.* 2019).

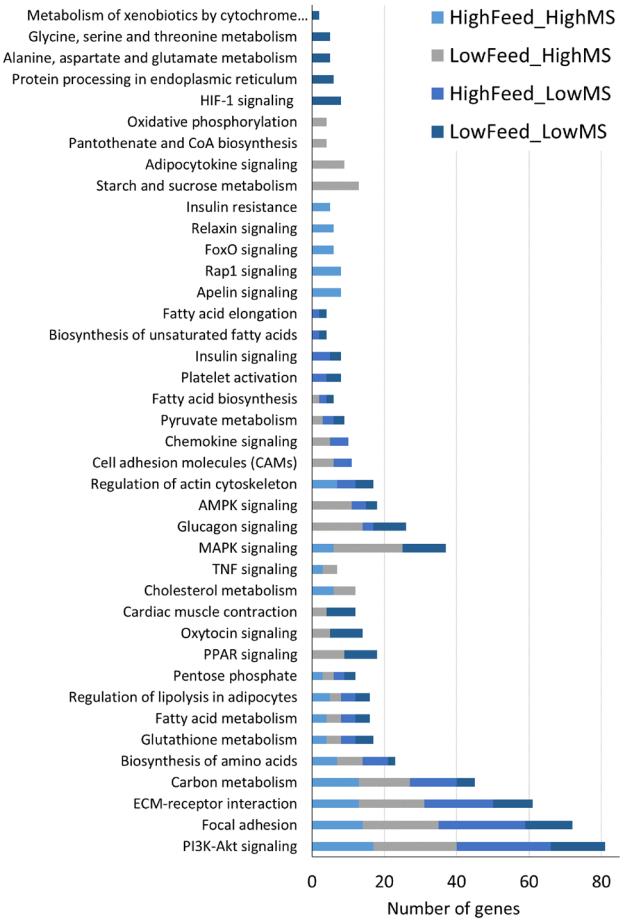

Figure 2. Number of genes involved in the pathways identified in each studied group

CONCLUSIONS

The co-expression analysis was shown to be a useful approach for the identification of processes and genes related to marbling development, particularly with the identification of gene modules that are associated with early age onset of marbling. The genes FOXO1, ADIPOR2, PGC-1α are promising markers to select for animals for high marbling.

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