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DEPOT-SPECIFIC GENE EXPRESSION DURING DIFFERENTIATION OF HANWOO MUSCULAR SATELLITE CELLS

S. De las Heras-Saldana[1], K. Y. Chung[2], S. H. Lee[3] and C. Gondro[1]

[1]School of Environmental and Rural Science, University of New England, Armidale, Australia
[2]Hanwoo Research Institute, National Institute of Animal Science, RDA, South Korea
[3]Division of Animal and Dairy Science, Chungnam National University, South Korea

SUMMARY

The Korean Hanwoo cattle is highly regarded for its high marbling ability (intramuscular fat) and this trait is the key drive of profitability for the industry. The study of muscle development and differentiation in Hanwoo is important to improve understanding of the genes and pathways that regulate these processes and to identify markers for genomic selection. In this study, we performed a culture cell experiment using bovine muscle satellite cells combined with a time-series RNA-seq analysis to measure the transcriptome expression levels during the development of satellite cells from *Longissimus dorsi* (LD) and *Semimembranosus* (SM) muscle. RNA-seq data was collected on days 0, 1, 2, 4, 7 and 14 after differentiation treatment with an average of 35,727,746 cleaned reads per sample. Between 77% and 85% of the reads were mapped to the reference genome (*Bos taurus* UMD3.1 from Ensembl). The genes Hoxc11, Sim2, Hoxc8, Hoxb9, Zic2, Zic4, Tbx4 and Hoxb4 were differentially expressed between LD and SM across time, suggesting that they could drive specific characterization of each muscle. The levels of expression vary vastly between time points according to the stage of muscle differentiation and development. At the beginning of the experiment, the genes involved in proliferation were enriched while their expression reduced drastically after day 2. However, at day 4 and until day 14 there was an enrichment in the genes involved in actin cytoskeleton, muscle cell differentiation and structural constituents of muscle.

INTRODUCTION

Biochemical, proteomic and gene expression characterization of the various muscle depots in cattle can assist our efforts to find improved markers for meat traits such as marbling or tenderness. The differentiation of bovine muscle satellite cells is a good model for muscle development studies since their nuclei contribute to postnatal muscle growth remodelling of pre-existing fibres and can provide insight into the genes involved in muscle growth and depot differentiation.

In muscle development, the Myogenic Regulatory Factors (MRFs) are well known to control myogenesis by the modulation of the myoblast proliferation, migration and fusion (Braun and Gautel 2011). There are four MRFs (Myf5, MyoD, Mrf4, and MyoG), however several other genes contribute to the balance of growth and differentiation (Eng *et al.* 2013). Genes for myogenesis (MYL2, MYH3) and adipogenesis (PPARϒ, and fabp4) of muscular satellite cells into myotubes-formed cells and adipocyte-like cells were identified in Hanwoo using microarrays (Lee *et al.* 2012).

Global RNA profiling of myogenesis in satellite cells is a good model to understand how changes in gene expression over time determine muscle proliferation and differentiation. However further work is needed to elucidate the molecular mechanisms involved in muscle differentiation and to understand differences between muscle types. In this study, two muscles (*Longissimus dorsi* -LD and *Semimembranosus* -SM) were sampled from three Hanwoo calves to extract muscle satellite cells (MSC). These cells were cultured and allowed to differentiate into myotubes, this process was studied using RNA-seq to characterize the transcriptional changes during myogenesis and how the gene expression profiles change between the differentiation of LD and SM.

MATERIALS AND METHODS

The satellite cells were isolated from the *Longissimus dorsi* (LD) and *Semimembranosus muscle* (SM) of three Korean Hanwoo new born calf as described previously by Frey *et al*. (1995) and Johnson *et al*. (1998). LD and SM satellite cells were cultured on DMEM containing DMEM and 10% FBS until they reached 60% to 70% confluence. We then induced differentiation using DMEM with 3% horse serum for 14 days. Cell samples were collected at confluence point (day 0) and at days 1, 2, 4, 7 and 14 after differentiation treatment to perform histological and RNA-seq analysis. The stain Hematoxylin and Hoechst was used to determine the muscle differentiation stages.

mRNA libraries were prepared and sequenced with the Illumina HiSeq 2000 sequencing system. The quality of resulting paired-end 100bp reads were assessed with FastQC v0.11.3, the adaptors and bad quality bases were removed with Trimmomatic v0.33. Bowtie2 v2.2.6 was used to map the reads to the reference genome *Bos taurus* (UMD3.1 from Ensembl). Bioconductor packages GenomicFeatures 1.22.13 and GenomicAlignments 1.6.3 performed the assembly and read count. We used edgeR 3.12.0 in the analysis of differentially expressed (DE) genes and considered significant DE genes those with a false discovery rate <0.05 and the logarithm fold change (logFC) ≥ 2. The functional enrichment of GO terms was performed by ClusterProfiler 2.5.5 and the pathway analysis was done with Pathview 1.10.1.

RESULTS AND DISCUSSION

The bovine MSC reached confluence approximately after 4 to 5 days of culture and it was at days 1, 2, after the differentiation treatment, when the myoblast initiated the terminal differentiation and multinucleated myotubes started to form, however they were not notably visible until day 4. In the last stage of differentiation myotubes went through significant morphological changes while they fused to form mature multinucleated myotubes.

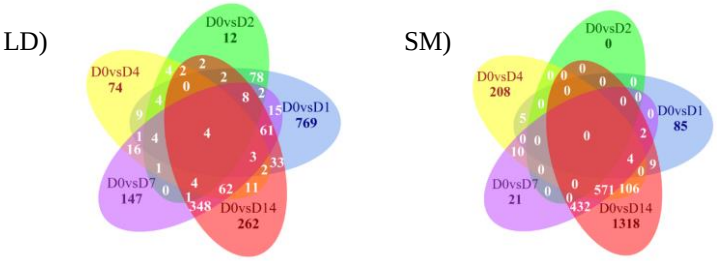

Figure 1. Number of common DE genes between time-point contrasts for LD and SM muscle.

From the sequencing data, between 77% and 84% of the reads were mapped (from average 35,727,746 total reads per sample) to the reference genome. We identified the genes that overlapped in each contrast of day 0 *versus* the sampled time and the genes that were differentially expressed exclusively at each time point (Figure 1). In LD, there were more DE at the beginning of the experiment with 769 DE genes expressed just in day 1, while the opposite pattern was observed in SM were there were 1318 DE genes at day 14. These results could reflect differences between muscle depots due to different rates of proliferation and differentiation during myogenesis.

The genes Hoxc11, Sim2, Hoxc8, Hoxb9, Zic2, Zic4, Tbx4 and Hoxb4 were differentially expressed between LD and SM at different time points, however some of these genes have not been previously directly associated with bovine muscle development.

Table I. GO functional annotation for the enriched terms in A) LD and B) SM muscle at days 1, 7, and 14. MF: molecular function; CC: cellular components; BP: biological process. Number of DE genes that are up-regulated (↑) and down-regulated (↓).

		d1 vs d0		d7 vs d0		D14 vs d0	
		Term	*DE*	*Term*	*DE*	*Term*	*DE*
LD	**MF**	Calcium ion binding	↓19	Cytoskeletal protein binding	↑7↓8	Receptor activity	↑15↓2
		Transmembrane receptor activity	↓15	Double-stranded DNA binding	↑4↓6	Cytoskeletal protein binding	↑9↓8
		Hormone activity	↑1↓7	Microtubule binding	↓7	Protein kinase binding	↑6↓5
						Peptidase inhibitor activity	↑4↓2
	CC	Extracellular space	↑ 4↓31	Cytoskeletal part	↑5↓20	Cytoskeletal part	↑7↓16
		Myofibril	↓25	Myofibril	↑12	Myofibril	↑13
		Sarcomere	↓22	Microtubule	↑12	Sarcomere	↑12
		Actin cytoskeleton	↓17	Sarcomere	↑11	Microtubule organizing center	↑1↓11
		I band	↓13	I band	↑7	I band	↑6
	BP	Regulation of multicellular organismal process	↑1↓37	Cell cycle	↑1↓33	Cell cycle	↑3↓29
		Immune system process	↑1↓27	Cytoskeleton organization	↑5↓15	Phosphorylation	↑13↓15
		Muscle structure development	↓17	Protein phosphorylation	↑6↓13	Cell proliferation	↑9↓16
		Actin filament-based process	↓14	Cell proliferation	↑5↓13	Muscle structure development	↑13↓3
		Muscle cell differentiation	↓11	Microtubule-based process	↓15	Negative regulation of proteolysis	↑6↓4
SM	**MF**			Cytoskeletal protein binding	↑12↓10	Receptor binding	↑15↓16
				Peptidase inhibitor activity	↑5↓4	Receptor activity	↑27↓3
				Protease binding	↑4↓2	Cytoskeletal protein binding	↑17↓12
	CC	Contractile fiber	↓10	Cytoskeletal part	↑23↓13	Extracellular space	↑34↓13
		Sarcomere	↓9	Extracellular space	↑24↓9	Cytoskeletal part	↑18↓24
		Myofibril	↓9	Myofibril	↑20	Myofibril	↑23↓1
		Actin cytoskeleton	↓9	Sarcomere	↑18	Sarcomere	↑21↓1
		I band	↓5	Microtubule	↑1↓12	I band	↑12
	BP	Muscle structure development	↓7	Cell cycle	↑5↓34	Immune system process	↑36↓10
		Regulation of muscle system process	↓5	Cytoskeleton organization	↑10↓16	Cell cycle	↑6↓36
		Striated muscle tissue development	↓5	Inflammatory response	↑12↓3	Cell proliferation	↑15↓23
		Regulation of muscle contraction	↓4	Muscle organ development	↑12	Cytoskeleton organization	↑16↓18
				G2/M transition of mitotic cell cycle	↑1↓4	Muscle structure development	↑21↓5

The Homeobox (HOX) family genes seem to be crucial for correct development and regulate muscle-specific genes (Houghton and Rosenthal 1999). In a study in mouse, the knockdown of Zic2 resulted in a delay in the activation of Myf5 with a subsequent delay in MyoD but the expression of Pax3 was not affected (Pan *et al*. 2011). Our results suggest that these eight genes could be involved in muscle type differentiation leading the muscle to develop characteristics specific for one depot or another.

With respect to the expression of the MRFs, we found that genes MyoG, MyoD and Myf6 showed higher expression in myoblasts in the process of differentiation and maintained high expression levels in myotubes which agrees with previous studies (Rajesh *et al*. 2011; Tripathi *et al*. 2014). At the protein level, Tripathi *et al*. (2014) reported that genes Myf5 and MyoD are highly expressed 10 days after differentiation treatment, however, in this study Myf5 did not present significant change in expression during differentiation.

In the enriched GO terms, we observed differences during the end of proliferation (day 0) compared with the myoblast differentiation (7 and 14 days after the differentiation treatments) (Table I). At the beginning of the experiment (day 0) the enriched terms were cell cycle, proliferation and G2/M transition of mitotic (Table I) in concordance with the proliferation events occurring in the myoblast. However, the expression of genes involved in these terms started to decrease in the subsequent days due to the shift from cell division to differentiation similar to previously reported in other bovine studies (Lee *et al*. 2012; He and Liu 2013). From the KEGG analysis some of the enriched pathways were dilated cardiomyopathy, cardiac muscle contraction, calcium signalling pathway, cell adhesion molecules (CAMs), cell cycle and adrenergic signalling in cardiomyocytes.

CONCLUSIONS

The differential expression of the genes Hoxc11, Sim2, Hoxc8, Hoxb9, Zic2, Zic4, Tbx4 and Hoxb4 suggest their implication in muscle depot differentiation during early development.

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